

ABSTRACT

Title of Dissertation: EXTRACELLULAR RNA IS TRANSPORTED
FROM ONE GENERATION TO THE NEXT
IN *C. ELEGANS*

Julia Ann Marré, Doctor of Philosophy, 2017

Dissertation directed by: Assistant Professor, Antony M. Jose, Department
of Cell Biology and Molecular Genetics

Experiences during the lifetime of an animal have been proposed to have consequences for subsequent generations. Although it is unclear how such intergenerational transfer of information occurs, RNAs found extracellularly in animals are candidate molecules that can transfer gene-specific regulatory information from one generation to the next because they can enter cells and regulate gene expression. In support of this idea, when double-stranded RNA (dsRNA) is introduced into some animals, the dsRNA can silence genes of matching sequence and the silencing can persist in progeny. Such persistent gene silencing is thought to result from sequence-specific interaction of the RNA within parents to generate chromatin modifications, DNA methylation, and/or secondary RNAs, which are then inherited by progeny. Here, we show that dsRNA can be directly transferred between generations in the worm *C. elegans*. Inter-generational transfer of dsRNA occurs even in animals that lack any DNA of matching sequence and dsRNA that reaches progeny

can spread between cells to cause gene silencing. Surprisingly, extracellular dsRNA can also reach progeny without entry into the cytosol, presumably within intracellular vesicles. Fluorescently labeled dsRNA is imported from the extracellular space into oocytes along with vitellogenin and accumulates in punctate structures within embryos. Subsequent entry into the cytosol of early embryos causes gene silencing in progeny. These results demonstrate the transport of extracellular RNA from one generation to the next to regulate gene expression in an animal and thus suggest a new mechanism for the transmission of experience-dependent effects between generations.

EXTRACELLULAR RNA IS TRANSPORTED FROM ONE GENERATION TO
THE NEXT IN *C. ELEGANS*

by

Julia Ann Marré

Dissertation submitted to the Faculty of the Graduate School of the
University of Maryland, College Park, in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
2017

Advisory Committee:

Assistant Professor Antony M. Jose, Chair

Associate Professor Kan Cao

Associate Professor Eric Haag

Professor Zhongchi Liu

Professor Kevin McIver

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Dedication

This dissertation is dedicated to my husband and my mother whose constant support and encouragement made my success possible.

Acknowledgements

This work was only possible because of the support and the guidance offered by my advisor, my lab-mates, my thesis committee, the other professors in the BISI department, the Baltimore Worm Club community, and my family.

I thank Dr. Antony Jose for helping me to learn how to be a scientist. Antony spent countless hours teaching me to effectively communicate science through writing and speaking, to think critically about a problem, and to focus on being precise and detailed in everything I do. Antony is and will be my role model as I embark on future endeavors. I am the scientist and the person I am today because of Antony.

I thank my lab-mates, past and present, for reading countless drafts of papers and grants in progress, for listening to me complain about failed experiments and for helping me make plans to solve those problems, and for the support and the friendship they have offered me. In particular, Pravrutha Raman, Sindhuja Devanapally, Snusha Ravikumar, and Nathan Shugarts have made every day in the lab a joy as I worked with these talented colleagues who were also my friends.

I thank Dr. Kan Cao, Dr. Zhongchi Liu, Dr. Kevin McIver, and Dr. Eric Haag for taking the time to be part of my thesis committee and for offering their constructive comments on my work. I especially appreciate the support my committee has offered me in these last few months while I have thought about my next steps post-graduate school and balancing my career and my family.

I thank the other professors in the BISI department at UMD for participating in departmental seminars and the members of the Baltimore Worm Club for offering feedback when I presented my work.

I thank my husband and my family for always being my cheerleaders and for taking turns watching baby Mirabelle while I wrote this dissertation.

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Chapter 1: General Introduction

1.1 What information is present in the zygote?

For many living things, each generation begins when two gametes combine to form a zygote. From this one cell, a multicellular organism develops, interacts with its environment, and generates gametes to produce the next generation. Thus, within the one cell of the zygote, all necessary information to enable development of the multicellular organism must be contained. Some carriers of information that we know are present in the zygote include DNA, parental mRNA and parental proteins, however, it is unknown if information from the parent's experiences reaches the zygote.

1.2 Ancestral experiences can affect descendants

The impact of ancestral experiences on descendants in diverse multicellular organisms has been evaluated and re-evaluated for more than a century. In 1801, Jean-Baptiste Lamarck proposed that experiences that affect an organism during its lifetime are passed on to offspring in his theory of inheritance of acquired traits (1). Charles Darwin later postulated in 1868 that packets of information that he named "gemmules" could be transported from the soma to the germline as a mechanism to explain the inheritance of acquired traits in his theory of pangenesis (2). However, in 1883, August Weismann proposed that experiences that affect an organism's somatic cells are unable to reach the germ cells and therefore unable to be inherited, which led to a concept called the Weismann's barrier (3). With the renewed interest in Gregory Mendel's work, the discovery of DNA, and the observation that in most animals the

germline separates from somatic cells early in development (reviewed in (4)), the theory of Weismann's barrier was embraced, while the theory of inheritance of acquired traits and the theory of pangenesis were abandoned. DNA was thought to be the sole carrier of inherited information. However, in recent work non-DNA sequence-based (or epigenetic) inheritance events have been described.

Both intergenerational and transgenerational effects have been observed in biology. In intergenerational effects, the parental experience can directly affect the germ cells of the offspring. In transgenerational effects, experiences affect the first generation (or F1) from the father or the third generation (or F3) from the mother (due to *in utero* effects).

Recent studies in animals have focused on changes in diet and stress as triggers in ancestors and found that such experiences correlate with changes in descendants (reviewed in refs. (5-8)). Changes in diet, for example, are correlated with mortality of grandprogeny in humans (9), altered metabolism of progeny in mice (10), and longevity of descendants in the worm *C. elegans* (11) (Fig. 1-1). Maternal separation (12), social defeat (13), and chronic variable stress (14) are correlated with hypersensitivity to similar stresses in descendants in mice. While these ancestral effects have been observed in many animals, for organisms where DNA sequence cannot be controlled between generations (i.e., the DNA sequence does not remain mostly constant between parents and progeny; e.g., non in-bred species or non self-progeny from hermaphrodites) the inheritance of a DNA sequence mutation cannot be easily distinguished from the inheritance of epigenetic effects.

Ancestral experiences can affect descendants in both plants and animals, but in animals, the germline cells are segregated early in development, while in plants, the germline cells arise later in development from somatic cells that have been exposed to environmental effects.

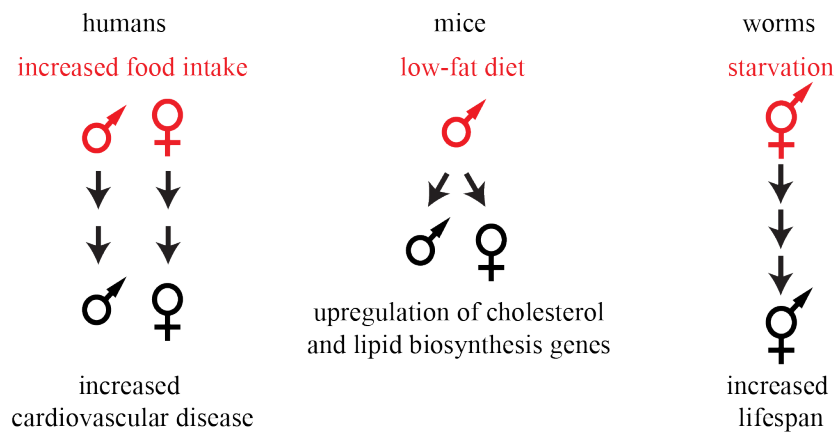


Fig. 1-1. Ancestral experience can cause changes in phenotypes observed in progeny. For example, changes in diet in humans, mice, and worms are correlated with altered disease states (9), metabolism (10), and lifespan (11), respectively.

1.3 Molecular candidates to transmit gene-specific information across generations

1.3.1 Requirements for signals that can be inherited

Molecules that transmit gene regulatory information from one generation to the next generation in response to the effects of diet or stress that somatic cells experience could provide a mechanistic explanation for the observed correlations. Molecules must reach the germline in the animal exposed to the environmental experience, must resist epigenetic reprogramming mechanisms, and must regulate specific genes in progeny to cause altered phenotypes (Fig. 1-2).

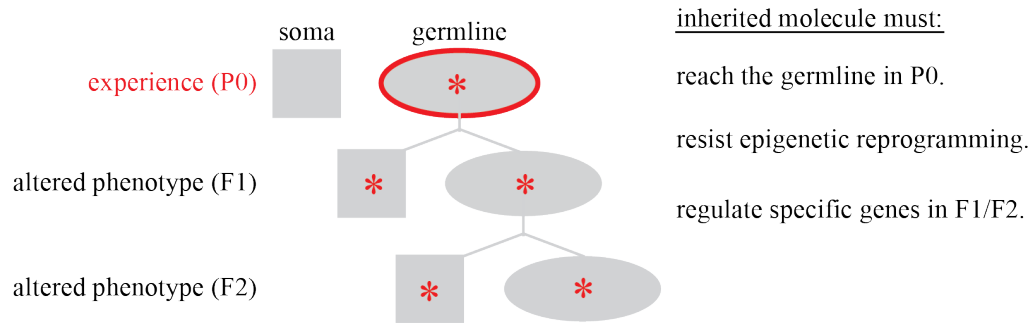


Fig. 1-2. An inherited molecule must reach the germline in the ancestor exposed to the experience, resist epigenetic reprogramming, and regulate specific genes in progeny. *, inherited molecule.

To reach the germline in animal cells, inherited molecules may need to cross cell membrane boundaries (e.g., molecules are secreted from somatic cells and then endocytosed into germline cells) or may not need to cross cell membrane boundaries (e.g., molecules from the cytosol of somatic cells bud out of somatic cells in an extracellular vesicle and fuse with the germline cell membrane to release molecules into the cytosol). Once molecules reach the germline, they must withstand reprogramming events that occur in the germline and post fertilization in the developing embryo such as DNA demethylation and exchange of histone variants.

1.3.2 DNA methylation

Methyl groups can be added to cytosine or adenine nucleotides in DNA. Cytosine methylation is the best understood and most commonly found methylation mark occurring mostly at CpG (Cytosine-phosphate-Guanine) sites in the DNA. These methylated regions are thought to contribute to the formation of heterochromatic regions in the genome and transcriptional silencing (reviewed in (15)). There are indications that DNA methylation may be a mark that is altered in

response to ancestral experiences as DNA methylation in mammalian and mouse genomes can be altered in the soma in response to environmental conditions (16, 17). Although in the germline of mammals, a significant portion of the genome undergoes the elimination of DNA methylation marks, there are regions that resist this reprogramming (18), suggesting that the persistence of DNA methylation marks through reprogramming could be a mechanism to alter the expression of specific genes in progeny in response to an ancestral experience.

1.3.3 Histone modifications

Genomic DNA is organized into nucleosome subunits consisting of DNA wrapped around a core of histone proteins. Histone proteins contain N-terminal amino acids tails that can be modified with groups such as acetyl, phosphoryl, and/or methyl. The type of modifications, if any, present on histone tails can impact the placement of histones on DNA and thus the genes accessible for transcription initiation (reviewed in (19)). Like DNA methylation, histone marks undergo significant reprogramming in the germline and post fertilization. For example, in the male germline, most histones are replaced with protamines thus eliminating histone modifications. However, if chromatin marks on the remaining histones are compromised, developmental defects can be observed in progeny (20, 21), suggesting that some histone modifications may resist reprogramming events and be present in progeny to alter specific genes.

1.3.4 Noncoding RNA

Noncoding RNAs can bind mRNA transcripts in a sequence-specific manner and initiate the degradation of mRNA and/or direct the placement of DNA

methylation and histone modifications on chromatin (reviewed in (5)). Noncoding RNAs can be detected within cells of the soma and of the germline and outside of cells in the extracellular space with or without membrane enclosure. RNAs in the germline are the best understood with diverse small noncoding RNA populations that associate with protein complexes (e.g., piRNAs with PIWI proteins) to maintain the integrity of the germline by directing chromatin marks to transposable elements or license the expression of specific germline genes (e.g., 22G-small RNAs associated with CSR-1 in *C. elegans*) (reviewed in (5)). Alterations in parental diet are correlated with altered small RNAs in *C. elegans* progeny (11) and with altered tRNA fragments in mouse sperm that affect metabolism genes in progeny (22, 23), suggesting that RNA populations can change in different environmental conditions to cause changes in progeny.

1.3.5 Extracellular RNA

Extracellular RNAs are candidates for transmitting gene-specific information from somatic cells to the germline and thus to the next generation because they can be detected in circulation (e.g., (24)), their composition is altered in disease states (e.g., (25)), and they can enter cells to regulate genes of matching sequence (e.g., (26)) (reviewed in (27)). While extracellular RNAs can be detected in human blood (24), saliva (28), breast milk (29), placenta (30), and semen (31), little is known about where these RNAs come from (e.g., in which cells can biogenesis occur?), where these RNAs can go (e.g., in what cells or tissues can they enter?), and what effects these RNAs can have within the cell (e.g., what specific genes can be affected?). Unraveling these questions surrounding extracellular RNAs are of particular interest

to human health as these RNAs could be used as biomarkers to diagnose disease states (32).

There is evidence that RNA, DNA methylation and histone modifications affect the presence of each other. RNAs can direct the deposition of DNA methylation and histone modification marks and DNA methylation and histone modification marks can direct the production of RNAs (reviewed in (5)). Some of these relationships may be to initiate and others may be to maintain the epigenetic effect that is inherited. To distinguish between initiation and maintenance mechanisms, it is essential to study a model where the ancestral experience can be introduced in a generation and the specific genes altered in progeny are known so their fate can be followed in subsequent generations.

1.4 Inheritance of RNAi in *C. elegans* as a model to study inherited effects of extracellular RNA

Studies in the worm *C. elegans* have provided some of the clearest evidence for RNA acting as a carrier of gene-specific information from somatic cells to germ cells in an animal. Expression of double-stranded RNA (dsRNA) in *C. elegans* neurons generates mobile RNAs that can silence a gene of matching sequence through RNA interference (RNAi) within the germline and this silencing can persist for more than 25 generations (33). Similar persistent silencing also occurs when dsRNA is delivered into worms by injection (34), soaking (35), or through expression within bacteria that worms ingest as food (36). Silencing of somatic genes typically

persists for one generation but silencing of germline genes can persist for many more generations (see Fig. 1-3 for summary of previous studies).

Extracellular RNA can be introduced to other organisms and cause silencing of specific genes in progeny (e.g., insects ((37))), but *C. elegans* is particularly well suited to study these effects. *C. elegans* has a generation time of ~3 days, facilitating multigeneration studies. *C. elegans* are hermaphrodites that produce both sperm and oocytes and lay self progeny, thus, *C. elegans* is a highly in-bred organism as self progeny from hermaphrodites are mostly genetically identical to parents. Rare males in the *C. elegans* population can be mated with hermaphrodites to produce cross progeny, enabling easy genetic manipulation.

gene type	tissue	target	% silenced (n)	# generations (method of RNAi)	ref.
multicopy transgene	ubiquitous	<i>Plet-858::gfp</i>	4 - 8 (50)	1''	146
		<i>Psur-5::sur-5::gfp</i>	98	1*	42
			100	1*	33
	muscle	<i>Pmyo-3::gfp</i>	100	1^	34
single copy transgene	germline	<i>Ppie-1::gfp::H2B</i> (in <i>eri-1(-) bkgd.</i>)	100 (10)	> 20*	100
			65 - 80 (5 - 18)	4*	102
		<i>Ppie-1::gfp::H2B</i>	60 - 100	9*	101
		<i>Pmex-5::gfp</i>	100 (> 14)	> 25`	33
endogenous	germline	<i>mex-3</i>	100 (615)	1*	38
			86 (665)	1^	38
		<i>mom-2</i>	40 (15)	2^	75
		<i>oma-1</i>	22 - 90 (3 - 6)	5*	101
		<i>pos-1</i>	50 (88)	2^	75
			64 (1564)	1*	101
		<i>sgg-1</i>	52 (23)	2^	75
	muscle	<i>unc-22</i>	100 (21)	1^	34
			100 (394)	1*	38
			68 (701)	1^	38
	skin	<i>dpy-11</i>	98	1*	42

Fig. 1-3. Published cases of silencing observed in self progeny when dsRNA against multicopy transgenes, single-copy transgenes, or endogenous target genes was introduced outside the germline in hermaphrodites. Method of RNAi: *environmental RNAi, ^injection into intestine or body cavity, `soaking RNAi, or `dsRNA expressed in tissues.

1.5 Silencing in *C. elegans*

Silencing by extracellular dsRNA requires entry of dsRNA into the cytosol, which is the aqueous component of the cytoplasm within which various organelles and particles are suspended. In all cases, entry of extracellular dsRNA into the cytosol of *C. elegans* cells requires the dsRNA-selective importer SID-1 ((38-40) and Fig. 1-4

A). Upon entry into the cytosol, dsRNA is processed to generate small RNAs that are used as guides to identify mRNA of matching sequence. The target mRNA is then used as a template to generate numerous secondary small RNAs that can direct the deposition of repressive chromatin marks (reviewed in ref. (41)). Although secondary small RNAs and chromatin marks have been detected in progeny upon parental exposure to dsRNA (42, 43), it is unknown where extracellular dsRNA needs to interact with intracellular RNA or DNA to cause gene silencing in progeny (Fig. 1-5).

1.6 Entry of extracellular dsRNA into the cytosol of cells in *C. elegans*

The import of extracellular dsRNA into the cytosol of cells requires the transport of dsRNA across the cell membrane. The systemic RNAi defective (or *sid*) screen in *C. elegans* identified several proteins required for the import of dsRNA into cells.

1.6.1 SID-1

SID-1 is a transmembrane protein that localizes to the cell membrane in non-neuronal cells (38) and is thought to assemble as a multimer to facilitate the transport of dsRNA into the cytosol of cells ((38, 40) and Fig. 1-4 A). SID-1 is conserved with homologs in all sequenced vertebrates and many invertebrates (Fig. 1-4 B) except for two-winged insects (e.g., *Drosophila*) (38, 44). Many of the experiments interrogating the role of SID-1 have been performed by expressing SID-1 in *Drosophila* S2 cells that do not contain a known SID-1 homolog. In these experiments, SID-1 has been shown to selectively transport dsRNA and not dsDNA or DNA:RNA hybrids across the cell membrane ((40) and Fig. 1-4 A) and dsRNA

uptake did not appear to be dependent on endocytosis or the use of ATP (39). However, *rsd-3*, the homolog of human Clathrin interactor 1, was also found to play a role in dsRNA import into cells (45), suggesting that endocytosis may be involved. *C. elegans* SID-1 can also enhance the uptake of dsRNA in mouse embryonic stem cells (46), in several insect cell lines (47-50), and in *C. elegans* neurons when expressed under a neuronal promoter (50). Using extrachromosomal arrays, *C. elegans* SID-1 tagged with GFP localizes to the cell membrane in all non-neuronal cells (38); however, the mammalian homolog localizes to the membrane of lysosomes (52). Because extrachromosomal arrays are silenced in the germline, it is currently unknown where SID-1 localizes in the *C. elegans* germline.

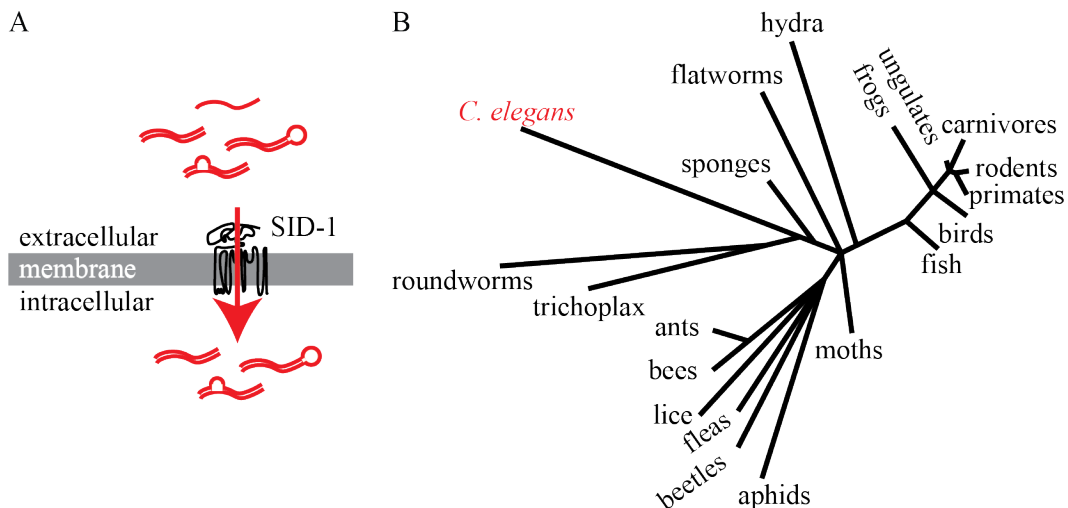


Fig. 1-4. dsRNA is imported into cells through the conserved transmembrane protein SID-1. (A) SID-1 selectively imports nucleic acids containing dsRNA domains. Adapted from (39). (B) SID-1 is highly conserved. Adapted from (44). Conservation based on sequence.

1.6.2 SID-2

SID-2 is a transmembrane protein located in the apical membrane of the intestine (53) and is thought to act as a receptor to transport ingested dsRNA from the lumen into the worm (54). While SID-2 is required for the import of ingested dsRNA into the worm, dsRNA already present within the worm (e.g., dsRNA injected into the body cavity) does not require SID-2 for entry into cells (53). SID-2 has homologs in some nematodes, but there is variability in the extracellular domains. *C. briggsae*, a close relative of *C. elegans*, is unable to uptake dsRNA from the environment and contains a SID-2 homolog with an extracellular domain that significantly varies from *C. elegans* SID-2 (53). However, if *C. elegans* SID-2 is expressed in *C. briggsae*, dsRNA can be taken up from the environment, suggesting that the presence of SID-2 determines whether a nematode is capable of environmental RNAi (i.e., silencing genes in an animal by ingesting dsRNA from the environment) (53).

1.6.3 SID-3

SID-3 is a tyrosine kinase that localizes to intracellular puncta in *C. elegans* and has homologs in many animals (55), including human activated Cdc42-associated kinase that promotes endocytosis (56), suggesting that SID-3 has a similar role in *C. elegans*.

1.6.4 SID-5

SID-5 is a transmembrane protein that partially colocalizes with endosomes and is thought to be involved in the transport of vesicles (57). SID-5 is thought to be required for uptake of dsRNA from the lumen to cause silencing in the intestine (57).

1.7 Nutrients that enter the germline and reach progeny

In this dissertation, I have analyzed how information enters the germline and reaches progeny. In addition to information, the zygote must contain nutrients to enable the development of the zygote. Yolk is one such nutrient present in the zygote of most egg-laying animals.

Yolk can be found in most egg-laying invertebrates and all egg-laying vertebrates and is primarily provided to progeny to be used as a nutrient source during embryogenesis (reviewed in (58)). Yolk is thought to primarily consist of a complex of yolk proteins and lipids. Vitellogenins, precursors to yolk proteins, are synthesized in a tissue outside the germline, secreted into circulation as lipoprotein complexes, and endocytosed into the ovary by receptor mediated endocytosis where processing occurs to form mature yolk proteins (reviewed in (58)). Vitellogenins are mainly expressed in the egg-producing sex (females or hermaphrodites) and are under many regulatory mechanisms to enable expression at stage-specific times (59). There are three vitellogenin gene families; vertebrate, *Drosophila*, and sea urchin. Vertebrate vitellogenins are also closely related to human apoB-100, an apolipoprotein component of mammalian LDL particles (60, 61).

1.7.1 Vitellogenin in vertebrates

Vitellogenins are transcribed in the liver in vertebrates (58). Vitellogenin proteins in vertebrates form dimeric lipoprotein complexes consisting of ~15-20% lipid. Vertebrate vitellogenins consist of a signal peptide, a heavy chain lipovitellin, a phosvitin, a light chain lipovitellin, and a von Willebrand factor type D domain (reviewed in (58)).

Endocytosed yolk platelets are rapidly sorted to early endosomes in the oocyte where the internalized vesicle is acidified. The acidic environment then activates cathepsin D protease, cleaving vitellogenins into lipovitellins, phosvitins, and the β' (derived from the von Willebrand factor type D domain) (reviewed in (62, 63)).

1.7.2 Vitellogenin in *C. elegans*

Vitellogenins are transcribed in the adult hermaphrodite intestine in *C. elegans* (64). There are six *vit* genes in *C. elegans*. *vit-1* to *vit-5* are located on the X-chromosome and *vit-6* is on chromosome IV, but only *vit-2*, *vit-5*, and *vit-6* are expressed. *vit-2* encodes yp170B, *vit-5* encodes yp170A, and *vit-6* encodes yp115/88 that is cleaved in the body cavity into two proteins (reviewed in (61)). These four vitellogenin proteins (yp170A, yp170B, yp115, and yp88) form two complexes (A complex and B dimer). The A complex is composed of yp170A, yp115, and yp88 and the B dimer is composed of two yp170B (65). These lipoprotein complexes are composed of ~8% phospholipids, 3% triglycerides and 3% other lipids (65). *C. elegans* vitellogenins share amino acid sequence homology with vertebrate vitellogenins and with apoB-100 (60, 61), but do not contain the phosvitin domain.

Vitellogenins in *C. elegans* are most closely related to vertebrate vitellogenins (61). Like in vertebrates, in *C. elegans*, vitellogenin is endocytosed into oocytes by receptor mediated endocytosis (66) and yolk platelets are processed in early embryos by a cathepsin protease (cathepsin L protease, CPL-1 (67)).

1.7.3 Organismal regulation of vitellogenin

In vertebrates, vitellogenin production is regulated by the hypothalamus-pituitary-gonad (HPG) axis involving hormonal signaling. Estrogen, in particular, can

bind receptors in the liver that initiate the production of vitellogenin (reviewed in (68)). In invertebrates, like *C. elegans*, most of the regulatory genes of vitellogenin production are unknown, but recently a *C. elegans* homeobox protein with homologs in mammals that mediate sexual differentiation and a putative transcription factor for vitellogenin production in the intestine were identified (69).

1.8 What signals can enter the germline and reach progeny?

Using RNAi in *C. elegans*, the goal of this dissertation is to determine how extracellular dsRNA can cause silencing in progeny (Fig. 1-5). Specifically, we are interested in identifying the initiating event that can be transmitted from parent to progeny (e.g., chromatin mark deposited in the parent germline that persists to progeny?).

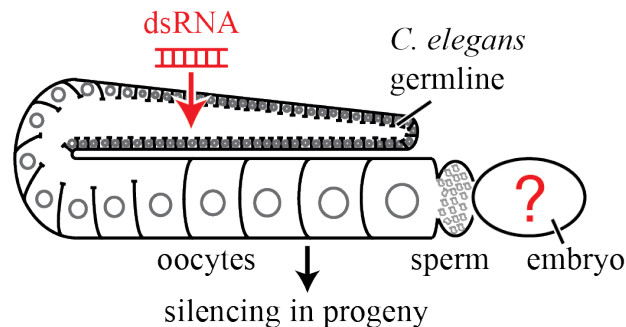


Fig. 1-5. It is unclear what signals can enter the germline and be transmitted through sperm or oocytes to be present in the embryo when dsRNA is introduced outside the germline. Schematic of one gonad arm of the *C. elegans* germline. Oocytes are made continuously in single-file order and then ovulate through the spermatheca where fertilization by sperm occurs.

Chapter 2: Genetic analyses suggest that extracellular RNA can be transmitted to progeny through oocytes

2.1 Preface

All the work presented in this chapter was published with some modifications as: Marré JA, Traver EC, and Jose AM (2016) Extracellular dsRNA is transported from one generation to the next in *C. elegans*. *Proceedings of the National Academy of Sciences USA* 113(44):12496-501.

Yinglun Wu performed the P0 RNAi experiment with *Pend-1::gfp* and imaged embryos to generate Fig. 2-2 D. Ed Traver performed the F1 RNAi experiments for RDE-1 and RDE-4 to generate Fig. 2-9 and Fig. 2-10. Pravrutha Raman performed the RNAi experiments for *dpy-7* and *act-5* to generate part of Fig. 2-11 B. Sindhuja Devanapally performed the neuronal dsRNA experiments to generate Fig. 2-12 C. All other data were generated by Julia Marré.

Some worm strains were obtained from the *Caenorhabditis elegans* Genetic stock Center, the Hunter laboratory (Harvard University), and the Seydoux laboratory (Johns Hopkins University). The Hamza laboratory (University of Maryland) provided some of the bacteria that express dsRNA.

2.2 Introduction

In this chapter, we begin interrogating how extracellular dsRNA can cause silencing in progeny by introducing dsRNA to worms by feeding worms bacteria that express dsRNA, by injecting dsRNA into the extracellular fluid, or by expressing dsRNA from within a somatic tissue. We use genetic mutants and rescues of essential

genes involved in RNA processing in specific tissues of the worm to infer the species of RNA that can be inherited and the route RNA travels from parent to progeny.

We found that the target gene does not need to be present in parents for silencing to occur in progeny and that the extracellular dsRNA itself can reach progeny. We restricted the entry of extracellular dsRNA into the cytosol and the processing of dsRNA within the cytosol by restricting the expression of the dsRNA importer SID-1 and the processing proteins RDE-4 and RDE-1 to specific tissues. These genetic analyses suggest that forms of dsRNA can reach progeny and can spread between cells to enable silencing.

2.3 Materials and methods

2.3.1 Strains, transgenesis, and oligonucleotides

All strains used are listed in Table 2-1 and all oligonucleotides used are listed in Table 2-2. Strains were cultured and maintained as described in (70).

Table 2-1. Strains used.

Strain	Genotype
AMJ8	<i>juIs73 [Punc-25::gfp] III</i>
AMJ12	<i>ccls4251 [pSAK2 (Pmyo-3::nlsGFP-LacZ) & pSAK4 (Pmyo-3::mitoGFP), & dpy-20] I [4x]</i>
AMJ180	<i>mIs10 [Pmyo-2::GFP, Ppes-10::gfp, PF22B7.9::gfp] V</i>
AMJ190	<i>oxSi221 [Peft-3::GFP + cb-unc-119(+)] II; rde-4(ne301)III</i>

AMJ256	<i>rde-4(gk884455) III</i>
AMJ286	<i>jamSi1 [Pmex-5::rde-4(+)] II; rde-4(ne301) III</i>
AMJ291	<i>oxSi221 II; unc-119(ed9) III; sid-1(qt9) V</i>
AMJ325	<i>oxSi221 II; unc-119(ed9) III; rde-1(ne219) V</i>
AMJ345	<i>jamSi2 [Pmex-5::rde-1(+)] II; rde-1(ne219) V</i>
AMJ542	<i>sid-1(qt9) V; jamEx140 [Prgef-1::gfp-hpRNA, Pmyo-2::DsRed]</i>
AMJ593	<i>oxSi487 dpy-2(e8) II; unc-119(ed3)? III; sid-1(qt9) V</i>
AMJ598	<i>oxSi487 dpy-2(e8) II; unc-119(ed3)? III; sid-1(qt9) V; jamEx140</i>
AMJ794	<i>unc-119(ed3) III (?); sid-1(qt9) V; telIs46 [Pend-1::gfp::H2B]</i>
AMJ843	<i>jamSi2 II; nrIs20 IV; rde-1(ne219) V</i>
EG4322	<i>ttTi5605 II; unc-119(ed9) III</i>
EG6070	<i>oxSi221 II; unc-119(ed9) III</i>
EG6787	<i>oxSi487 II; unc-119(ed3) III</i>
HC195	<i>nrIs20 [Psur-5::sur-5::gfp::unc-54 3' utr] IV</i>
HC196	<i>sid-1(qt9)</i>
HC738	<i>nrIs20 IV; rde-1(ne219) V</i>
JH3197	<i>K08F4.2(ax2053[K08F4.2::gfp])</i>
N2	wild type
WM27	<i>rde-1(ne219) V</i>
WM49	<i>rde-4(ne301) III</i>

Table 2-2. Oligonucleotides used (5' to 3', IDT).

Oligonucleotide	Sequence
P1 (Pmex-5::rde-4 fwd)	gatccaactcctcgacgcac
P2 (Pmex-5::rde-4 rev)	cgtagtttggttaaatccattctctgtctgaaacattc
P3 (Pmex-5::rde-4 fwd_nested)	tctctcactagtagtccgcagagacaaccatc
P4 (rde-4 fwd)	gaatgtttcagacagagaatggatttaaccaaactaacg
P5 (rde-4 rev)	cactgcagagaatgagtg
P6 (rde-4 rev_nested)	gagagaactagtagaggtcagagggcatag
P7 (Pmex-5::rde-1 fwd)	agtcagtgcgcgaggaagc
P8 (Pmex-5::rde-1 rev)	tcgggaaaattcgaggacattctctgtctgaaacattcaatt
P9 (Pmex-5::rde-1 fwd_nested)	tgtaaacgacggccagt
P10 (rde-1 fwd)	aattgaatgtttcagacagagaatgtcctcgaattttccga
P11 (rde-1 rev)	tcacacttctccagttgagc
P12 (rde-1 rev_nested)	gagagacctgcaggggaagtcgtgaaatcacctgc
P13 (Pmex-5 genotype fwd)	ccgtactccgtttgtttgatc
P14 (rde-4 genotype rev)	tcgggaaggcttcataaggaac
P15 (rde-1 genotype rev)	tgccgtcgcatttaccagtg
P16 (T7 fwd)	taatacgactcactataggg

To express RDE-4 in the germline (*Pmex-5::rde-4(+)*): The promoter for *mex-5* (*Pmex-5*) was amplified (Phusion polymerase, NEB) from N2 genomic DNA (gDNA) using the primers P1 and P2. The *rde-4* gene was amplified (Phusion polymerase, NEB) from N2 gDNA using the primers P4 and P5. Using these two amplicons as template, *Pmex-5::rde-4(+)* was generated (Phusion polymerase, NEB)

with primers P3 and P6. This final product (*Pmex-5::rde-4(+)*) was purified (QIAquick PCR Purification Kit, Qiagen) and cloned into pCFJ151 using the *SpeI* (NEB) restriction site to generate pJM1. pJM1 (22.5 ng/μl) and the coinjection markers pJL43.1 (50 ng/μl), pMA122 (10 ng/μl), pGH8 (10 ng/μl), pCFJ90 (2.5 ng/μl), and pCFJ104 (5 ng/μl) (plasmids described in (71)) were injected into the germline of adult EG4322 animals. One transgenic line was isolated as described earlier (71) and crossed into *rde-4(ne301)* animals to generate AMJ286. The integration of *Pmex-5::rde-4(+)* in AMJ286 was verified by genotyping AMJ286 using primers P13 and P14.

To express RDE-1 in the germline (*Pmex-5::rde-1(+)*): The promoter for *mex-5* (*Pmex-5*) was amplified (Phusion polymerase, NEB) from pJA252 (Zeiser) using the primers P7 and P8. The gene *rde-1* was amplified (Phusion polymerase, NEB) from N2 gDNA using the primers P10 and P11. Using these two amplicons as template, *Pmex-5::rde-1(+)* was generated (Phusion polymerase, NEB) with primers P9 and P12. This final product (*Pmex-5::rde-1(+)*) was purified (QIAquick PCR Purification Kit, Qiagen) and cloned into pCFJ151 using the *AflII* and *SpeI* (NEB) restriction sites to make pJM2. The pJM2 plasmid (22.5 ng/μl) and the coinjection markers pJL43.1 (50 ng/μl), pMA122 (10 ng/μl), pGH8 (10 ng/μl), pCFJ90 (2.5 ng/μl), and pCFJ104 (5 ng/μl) (plasmids described in (Zeiser)) were injected into the germline of adult EG4322 animals. One transgenic line was isolated and crossed into an *rde-1(ne219)* background to make AMJ345. The integration of *Pmex-5::rde-1(+)* in AMJ345 was verified by genotyping AMJ345 using primers P13 and P15.

2.3.2 Feeding RNAi

Control RNAi by feeding *E. coli* containing the empty dsRNA-expression vector (pL4440), which does not produce dsRNA against any gene, was done in parallel with all RNAi assays and all silencing defects were scored (Table 2-3) in comparison to that observed (if any) upon pL4440 feeding.

Table 2-3. Scoring of gene-specific silencing.

Gene	Site expressed	Defect scored upon RNAi
<i>bli-1</i>	hypodermis	Presence of fluid-filled blisters on adults
<i>div-1</i>	germline	Dead (unhatched) eggs
<i>dpy-2</i>	hypodermis	Short, fat L4 animals
<i>dpy-7</i>	hypodermis	Short, fat L4 animals
<i>fkh-6</i>	somatic gonad	Dead (unhatched) eggs
<i>gfp</i>	muscle	L4-staged worms had dimmed or absent GFP
	intestine	expression when viewed using Olympus fluorescent
	ubiquitous	scope compared to worms fed control RNAi food.
	germline	
<i>sqt-3</i>	hypodermis	Adults that roll
<i>let-858</i>	germline	Dead (unhatched) eggs
<i>par-1</i>	germline	Dead (unhatched) eggs
<i>pos-1</i>	germline	Dead (unhatched) eggs
<i>unc-15</i>	body-wall muscle	Slow/lethargic movement of L4 animals
<i>unc-22</i>	body-wall muscle	Weak: L4 or young adults occasionally twitch within 1

minute in response to 3 mM levamisole (Sigma Aldrich).

Strong: L4 or young adults continuously twitch within 1 minute in response to 3 mM levamisole (Sigma Aldrich).

unc-52 hypodermis Slow/lethargic movement, including paralysis, of L4 animals

Inheritance assay in response to P0 RNAi: RNAi bacteria was grown in LB-carbenicillin overnight and 100 µl was seeded on RNAi plates (NG agar plate supplemented with 1 mM IPTG (Omega) and 25 µg/ml carbenicillin (MP Biochemicals)). Seeded RNAi plates were incubated at room temperature for 1-2 days before L4-staged worms were added. The plates were then incubated at 20°C for one day. RNAi bacteria were then removed in one of the following ways:

4x wash: The RNAi fed worms were suspended in 1 ml of M9 buffer in a 1.5 ml microcentrifuge (VWR) tube and spun at 8,000 rpm for 30 seconds. After removing 800 µl of the old buffer, an equal volume of fresh M9 buffer was added. This washing was repeated four times and the final 200 µl of M9 buffer with worms was placed on plates seeded with OP50 and incubated for one hour at room temperature before each worm was moved to a fresh plate seeded with OP50.

Bleach: The RNAi fed worms were placed into a small drop of 0.6% NaOCl (10% of Chlorox®) in 1.5M NaOH (Sigma-Aldrich) on individual OP50 seeded agar plates.

Kanamycin: The RNAi fed worms were washed with buffer as described above for “4x wash” and then placed onto individual NG-kanamycin plates (50 µg/ml kanamycin (EMD Millipore)) seeded with 100 µl OP50. Plates were checked each day for remaining OP50 and more OP50 was added if needed.

For all of the above RNAi bacteria removal methods, the earliest L4-staged progeny were scored (~20 per worm) for inherited gene silencing by assaying gene-specific effects (typically two to three days later).

Silencing assay in response to F1 RNAi: A single L4-staged animal (P0) was placed on an RNAi plate (NG agar plate supplemented with 1 mM IPTG (Omega) and 25µg/ml carbenicillin (MP Biochemicals)) seeded with 5 µl of OP50 *E. coli* and allowed to lay eggs. After one day, the P0 animal was removed, leaving the F1 progeny embryos. 100 µl of an overnight culture of RNAi food (*E. coli* that express dsRNA against a gene of choice) was added to the plate. The earliest L4-staged progeny were scored for gene silencing by assaying gene-specific effects (Table 2-3). For F1 RNAi of males, the starting P0 was a single gravid adult-staged animal from a mating plate that was started with three L4-staged hermaphrodites and nine males.

2.3.3 Balancing loci

Integrated transgenes expressing *gfp* were used to balance mutations in heterozygous animals. Progeny of heterozygous animals were scored as homozygous

mutants if they lacked both copies of the transgene. The *rde-4(ne301)* allele on Chr III was balanced by *juIs73* or *otIs173*. About 99% (153/155) progeny of *rde-4(ne301)/juIs73* that lacked fluorescence were found to be homozygous *rde-4(ne301)* animals either by Sanger sequencing or by resistance to *pos-1* RNAi. The *rde-1(ne219)* and *sid-1(qt9)* alleles on Chr V were balanced by *mIs10*. About 94% (63/67) progeny of *rde-1(ne219)/mIs10* that lacked fluorescence were found to be homozygous *rde-1(ne219)* by Sanger sequencing. The *jamSi1* and *jamSi2* alleles integrated into the *ttTi5605* Mos site on Chr II were balanced by *oxSi221*, which is a transgene that is also integrated at the *ttTi5605* Mos site on Chr II. Worms homozygous for *juIs73* or *oxSi221* were brighter than worms hemizygous for *juIs73* or *oxSi221* and could be reliably distinguished. For *otIs173* and *mIs10*, homozygous transgenic animals could not be distinguished from hemizygous animals and were thus grouped together (i.e., ++ and +/- genotypes for *rde*).

2.3.4 Injection of dsRNA

unc-22 dsRNA: The *unc-22* sequence with flanking T7 promoters was amplified (Phusion polymerase, NEB) from the *unc-22* RNAi vector using the P16 primer. The product was purified (QIAquick PCR Purification Kit, Qiagen) and dsRNA was transcribed in vitro (T7 High Yield RNA Synthesis Kit, NEB). Transcribed dsRNA product was purified (QIAquick PCR Purification Kit, Qiagen), treated with RNase A (Omega Bio-Tek), and purified (QIAquick PCR Purification Kit, Qiagen).

Injection: Adult animals (24 hours post L4-stage) were injected with 159 ng/μl *unc-22*-dsRNA into the body cavity past the bend of the posterior arm of the

gonad (Fig. 2-12 and Fig. 2-13) or with 159 ng/μl *unc-22*-dsRNA into both arms of the germline (Fig. 2-7 D). Hermaphrodites injected with *unc-22*-dsRNA were crossed with males that express *gfp* to distinguish self and cross progeny. The earliest progeny or both early progeny that were L4-staged 3 days after injection and late progeny that were L4-staged 4 days after injection were scored (Fig. 2-7 D) for silencing of *unc-22*.

2.3.5 Fluorescence imaging

RNAi fed worms: Fourth-larval stage (L4) animals in 3 mM tetramisole hydrochloride (Sigma) were individually imaged at fixed magnification on an AZ100 microscope (Nikon) with a Cool SNAP HQ² camera (Photometrics). A C-HGFI Intensilight Hg Illuminator was used to excite GFP (filter cube: 450-490 nm excitation, 495 dichroic, and 500-550 nm emission). Exposure times were scaled for control RNAi fed worms to just under saturation for each genetic background and then *gfp* RNAi fed worms were imaged using the same exposure time. Corresponding bright-field images were taken using auto-exposure. Images were adjusted for display using ImageJ (NIH).

2.4 Results

2.4.1 Silencing signals are transported to progeny through oocytes

To evaluate gene silencing in progeny upon ingestion of dsRNA, we fed worms bacteria that express dsRNA, removed the bacteria, and examined silencing in progeny (Fig. 2-1 A). Using this assay, all silencing of endogenous genes as well as

transgenes detected in progeny was due to the inheritance of a silencing signal from parents to progeny (Fig. 2-2 and Fig. 2-3). We found that ingestion of dsRNA by animals from hatching until their fourth larval (L4) stage resulted in silencing in only ~10% of progeny, but, ingestion beyond the L4 stage for a 24-hr period resulted in silencing in ~100% of progeny (Fig. 2-1 *B*). Silencing occurred in all animals among early progeny but was observed in progressively fewer animals among later progeny (Fig. 2-1 *C*), as is the case when limiting amounts of dsRNA are introduced by injection into the germline (72). This reduction of inherited silencing is consistent with the dilution of silencing signals by two known processes: cytoplasmic streaming within the germline (73) and the flow of material from the intestine into oocytes (e.g., yolk (66)). Such dilution is expected to be progressive in oocytes, which are made continuously during adulthood, but not in sperm, which are made in a single batch during the fourth larval stage (Fig. 2-4, (74)). Furthermore, unlike the ~100% silencing that could be observed in progeny of hermaphrodites that ingested dsRNA, silencing was not detectable in any progeny of males that ingested dsRNA (Fig. 2-1 *D*) despite the detection of SID-1-dependent silencing within the germline of male parents (Fig. 2-1 *D* and Fig. 2-5). Together, these results suggest that ingested dsRNA or dsRNA-derived silencing signals that can be progressively diluted are transported to progeny through oocytes.

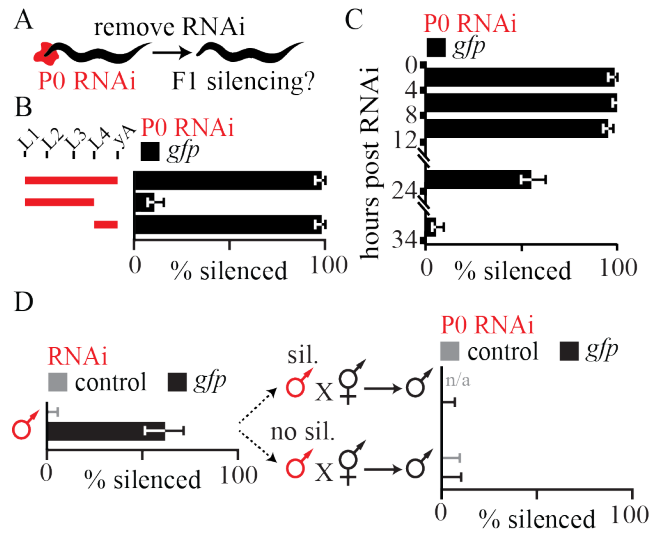


Fig. 2-1. Ingested dsRNA or dsRNA-derived silencing signals can be transported to progeny through oocytes. (A) Schematic of assay to assess silencing in progeny (F1) by parental ingestion of dsRNA (P0 RNAi). Also see Fig 6. (B and C) Silencing of multi-copy *gfp* transgenes in progeny by ingested dsRNA. (B) Robust silencing of *Psur-5::sur-5::gfp* in intestinal cells required parental ingestion of *gfp*-dsRNA during adulthood (L1 to L4 – larval stages; yA – young adult). (C) Silencing of *Pmyo-3::gfp* in muscle cells after parental ingestion of *gfp*-dsRNA was detectable in all early progeny (0-12 hours post RNAi) but only in diminishing fractions of later progeny (12-34 hours post RNAi). (D) Males showing silencing of *gfp* (*Pgtbp-1::gtbp-1::gfp*) within the germline (sil.) did not transmit silencing to any cross progeny (right). Males fed control RNAi did not show any silencing (n/a, not applicable). Error bars indicate 95% CI; L4-staged animals were assayed (n > 80 (B), n > 84 (C), n > 40 (D)); and grey bars indicate silencing in progeny of animals that ingested control dsRNA (D).

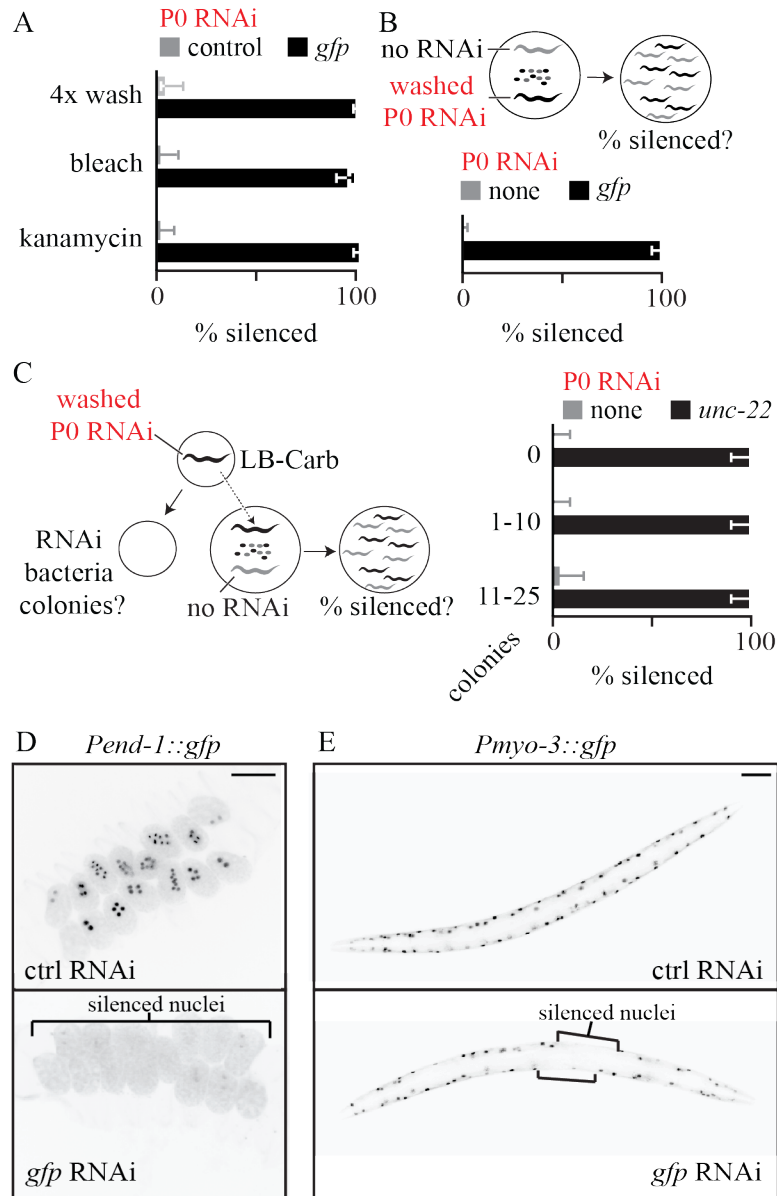


Fig. 2-2. Washing worms that ingested bacteria expressing dsRNA is sufficient to ensure that silencing in progeny is caused by inheritance of a silencing signal from parents and not by the ingestion of dsRNA by progeny. (A) Silencing (% silenced) of a multicopy *gfp* transgene (*Psur-5::sur-5::gfp*) in intestinal cells of progeny was detectable when the bacteria expressing *gfp*-dsRNA (P0 RNAi) was removed from parents by washing four times with buffer (4x wash) or by killing (bleaching P0 animals or placing animals on kanamycin plates). (B) A worm that was fed bacteria that express *gfp*-dsRNA and subsequently washed with buffer (washed P0 RNAi, black) was placed along with a worm that was not fed dsRNA but was marked with pharyngeal *gfp* expression (no RNAi, grey) and progeny from both worms were assessed for silencing of a multicopy *gfp* transgene (*Pmyo-3::gfp*) in muscle cells (left). Silencing (% silenced) was detectable only in muscle cells of progeny from parents fed *gfp*-dsRNA (P0 *gfp* RNAi, black bar) (right). (C) Wild-type

animals that were washed as in (A) after being fed carbenicillin-resistant RNAi bacteria (washed P0 RNAi, black) were allowed to crawl on carbenicillin plates for 1 hr before being cultured along with worms marked with a fluorescent marker, and the carbenicillin plates were incubated overnight to identify colonies generated by any residual bacteria that were not removed by the washes (RNAi bacteria colonies) (top). Although washing parent worms fed P0 RNAi did not eliminate a few RNAi bacteria in some cases (5/15 plates had 0 colonies; 5/15 plates had 1-10 colonies; and 5/15 plates had 11-25 colonies), silencing (% silenced) of an endogenous gene (*unc-22*) was detectable only in progeny from parents fed *unc-22*-dsRNA (P0 *unc-22* RNAi, black bars) and not in the co-cultured fluorescently marked worms (no RNAi, grey) in all cases (bottom). (D) Ingestion of bacteria that express *gfp*-dsRNA by animals that express a *gfp* transgene in intestinal cells (*Pend-1::gfp*, black circles) caused silencing in embryos held in utero. Data generated by Ying Wu. (E) Representative developed progeny from animals that ingested control dsRNA (top) or from those that ingested *gfp*-dsRNA showing silencing in muscle cells (regions within square brackets) of a multi-copy *gfp* transgene (*Pmyo-3::gfp*). Error bars indicate 95% CI (A-C), L4-staged animals were assayed (n > 46 (A), n > 104 (B), n > 50 (C)), and scale bars = 50 μ m (D and E). Grey bars are progeny from parent worms fed control RNAi (A) or no RNAi (B and C). Bleaching gravid adult worms only allows analysis of the few progeny embryos that are protected by their eggshell and held in utero. These results establish serial washing as a viable alternative.

gene type	tissue	target	% silenced (n)
multicopy transgene	gut	<i>Pend-1::gfp::H2B</i>	100 (50)
	muscle	<i>Pmyo-3::gfp</i>	98 (206)
	ubiquitous	<i>Psur-5::sur-5::gfp</i>	93 (205)
single copy transgene	ubiquitous	<i>Peft-3::gfp</i>	97 (201)
endogenous	muscle	<i>unc-22</i>	100 (50)
	skin	<i>dpy-7</i>	80 (81)

Fig. 2-3. Cases where silencing was observed in self progeny when using the inheritance assay. Hermaphrodites ingested dsRNA against multicopy transgenes, single-copy target transgenes, or endogenous target genes using the inheritance assay described above and in materials and methods.

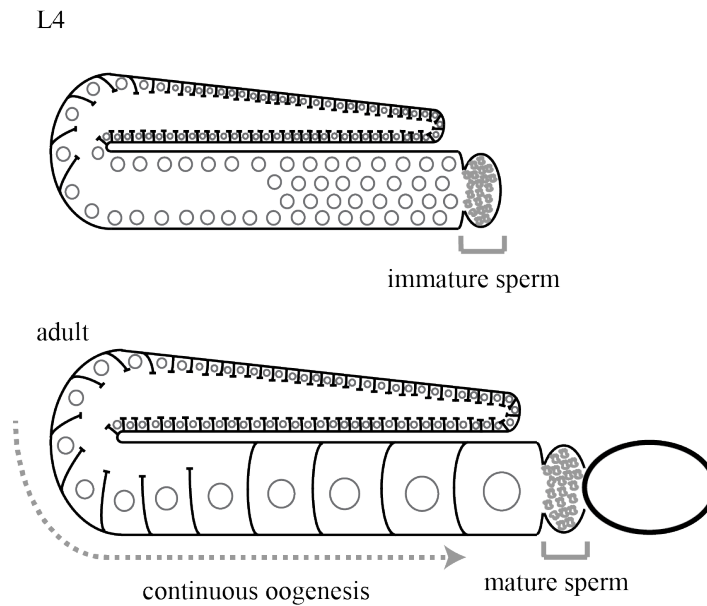


Fig. 2-4. Schematic of gamete production in *C. elegans*. Sperm is made in one batch within the gonad during the L4-stage (*top*) while oocyte production begins after the L4-stage and continues throughout adulthood (*bottom*).

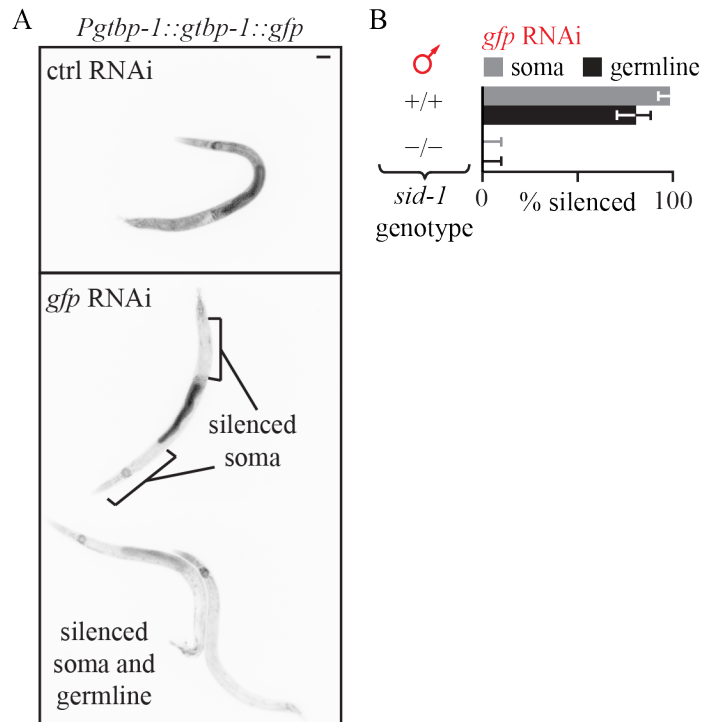


Fig. 2-5. Silencing of the male germline is dependent on SID-1 but is not detectable in all males that ingest *gfp*-dsRNA. (A) Ingestion of bacteria that express *gfp*-dsRNA (*gfp* RNAi) by animals with *Pgtbp-1::gtbp-1::gfp* caused silencing within the germline in some male animals (bottom two animals in lower panel) but not in others (top animal in lower panel). (B) Silencing (% silenced) of *gfp* fused to a genomic locus (*Pgtbp-1::gtbp-1::gfp*) in the somatic cells (grey bars) and in the germline (black bars) of males that ingested *gfp*-dsRNA was dependent on the presence of *sid-1*. Error bars indicate 95% CI; L4-staged animals were assayed (n > 43 (A)); and scale bar = 50 μ m (B).

2.4.2 Silencing in progeny does not require parents that ingest dsRNA to have DNA of matching sequence

The progressive dilution of silencing in progeny is consistent either with the inheritance of small RNAs synthesized using mRNA templates as was previously proposed in response to injected dsRNA (75) and ingested dsRNA (42) or with the inheritance of ingested dsRNA or its derivatives independent of any homologous sequence. To test if homologous sequences are required in animals that ingest dsRNA

for silencing in progeny, we exposed hermaphrodites lacking target sequences that match ingested dsRNA and examined silencing in cross progeny after introducing the target sequence through males. Silencing was detected in ~100% of progeny when *gfp*-dsRNA was ingested by hermaphrodite animals lacking a *gfp* transgene (Fig. 2-6). Thus, for ingested dsRNA to cause silencing of a matching gene in progeny, that gene need not be present in the parent that ingests the dsRNA.

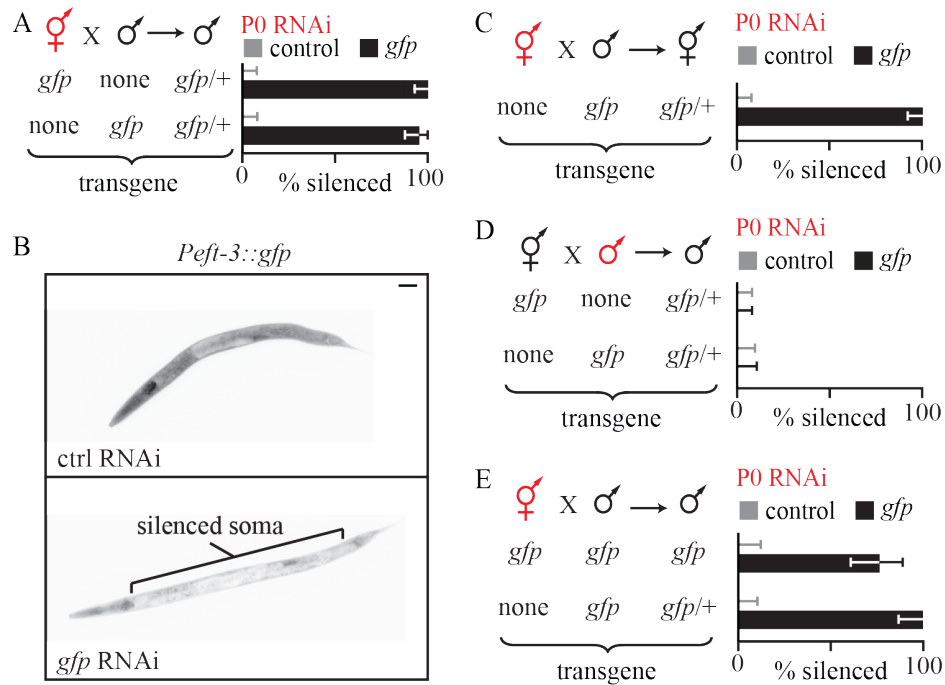


Fig. 2-6. Ingested dsRNA does not require matching DNA in parents to silence genes in progeny. (A-C) Silencing of a single-copy *gfp* transgene (*Peft-3::gfp*) in cross progeny was detected in somatic cells even when *gfp*-dsRNA was ingested by hermaphrodites that lack *gfp*. (A and C) Data for male (A) and hermaphrodite (C) cross progeny. (B) Representative images of cross progeny for data shown in A. Scale bar = 50 μ m (D) Silencing (% silenced) of a single-copy *gfp* transgene (*Peft-3::gfp*) in all somatic cells in cross progeny was not detected when males (red) that either lacked the *gfp* transgene (none) or that had the *gfp* transgene ingested *gfp*-dsRNA (black bars). (E) Silencing (% silenced) of a multicopy *gfp* transgene (*Pmyo-3::gfp*) in cross progeny was detected in muscle cells even when hermaphrodites (red) that lacked *gfp* (none) ingested *gfp*-dsRNA (black bars). Error bars indicate 95% CI; L4-staged animals were assayed (n > 56 (A), n > 56 (C), n > 47 (D), n > 48 (E)); and grey bars are as in Fig. 2-1.

2.4.3 Forms of dsRNA reach progeny and spread between cells in the embryo

The simplest hypothesis explaining the ability of ingested dsRNA to cause silencing in progeny even when parents lack matching sequences is that either the ingested dsRNA itself or a processed derivative is delivered into progeny. Processing of dsRNA can begin upon entry of dsRNA into the cytosol through SID-1 (Fig. 2-7 and reviewed in ref. (41)). The dsRNA is bound by the dsRNA-binding protein RDE-4 and recruited to the endonuclease Dicer, which processes it into primary double-stranded short-interfering RNAs (1° ds-siRNAs). One of the strands of 1° ds-siRNAs is eliminated by the Argonaute RDE-1 to generate primary single-stranded short-interfering RNAs (1° ss-siRNAs), which are used as guides to identify mRNAs of matching sequence. Subsequent recruitment of RNA-dependent RNA polymerases generates numerous secondary small RNAs (2° ss-siRNAs), which are used for silencing. Thus, while secondary RNAs require mRNAs of matching sequence for synthesis, all primary RNAs can be made independent of any homologous sequence.

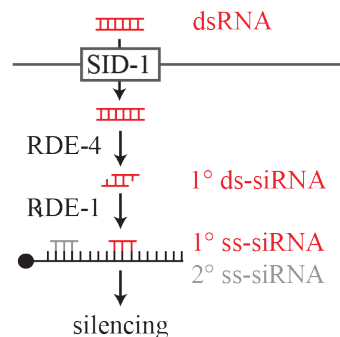


Fig. 2-7. Model of RNA silencing in *C. elegans*. Extracellular long dsRNA (red) enters the cytosol of cells through SID-1 and is processed by proteins (RDE-4 and RDE-1) into primary RNA species (1°-ds-siRNA and 1°-ss-siRNA, red) that are used to find target mRNA and trigger the synthesis of secondary RNA species (2°-ss-siRNA, grey), which results in gene silencing.

To determine the requirements for dsRNA processing in parent and in progeny to silence genes in response to ingested dsRNA, we examined silencing when SID-1, RDE-4, or RDE-1 were each present in either the parent or the progeny (Fig. 2-8, Fig. 2-9, and Fig. 2-10).

We found that presence of SID-1 in parents was not sufficient for silencing in progeny when only progeny ingested dsRNA (Fig. 2-8 *A* and *B*), suggesting that parental SID-1 does not persist in larval progeny to enable the import of ingested dsRNA. On the other hand, presence of SID-1 in parents was sufficient for silencing in progeny when only parents ingested dsRNA (Fig. 2-8 *E*), suggesting that entry of dsRNA into cells in parents or during early development in progeny is sufficient for silencing in progeny.

We found that presence of RDE-4 in parents was sufficient for silencing genes expressed in somatic tissues of progeny (Fig. 2-8 *F*), as noted for injected dsRNA in early experiments (76). Unlike in the case of SID-1, however, presence of RDE-4 in parents enabled silencing in progeny when progeny ingested dsRNA as larvae (Fig. 2-8 *C*). Silencing was robust for somatic genes, but undetectable for germline genes (Fig. 2-9), consistent with the failure to detect any maternal rescue of RDE-4 when dsRNA against germline genes was injected into progeny (75). Silencing of somatic genes, however, could be detected even when progeny only began ingesting dsRNA ~54 hours after egg-laying (Fig. 2-10 *C*), but could not be enabled by grand-parental RDE-4 (Fig. 2-10 *A* and *B*), consistent with the persistence of parental RDE-4 in progeny. Thus, detectable silencing in progeny when an animal ingests dsRNA matching a somatic gene requires entry into the cytosol in the animal that ingests the

dsRNA or during early development of its progeny; subsequent processing by RDE-4 can occur even in late-staged progeny. Similar experiments using RDE-1 revealed that when an animal ingests dsRNA matching a somatic gene, processing by RDE-1 must occur in that animal or during early development of its progeny for silencing in progeny (Fig. 2-8 *D* and *G*), consistent with observations using injected dsRNA (76).

In summary, analysis of *sid-1* mutants revealed that the entry of dsRNA into cells in parents or during early development in progeny is sufficient for silencing in progeny (Fig. 2-8 *A*, *B*, and *E*). Analysis of *rde-4* mutants revealed that recruitment of ingested dsRNA into the RNAi pathway can occur in animals that ingest dsRNA or in their progeny at any stage during development (Fig. 2-8 *C* and *F*, Fig. 2-9, Fig. 2-10, and (75, 76)). However, analysis of *rde-1* mutants revealed that the production of 1° ss-siRNAs must occur in animals that ingest dsRNA or in their progeny before larval development for silencing in progeny (Fig. 2-8 *D* and *G* and (76)). Therefore, ingested dsRNA and all primary RNAs derived from it can be processed in the animal that ingests dsRNA or in its progeny during early development for silencing in progeny.

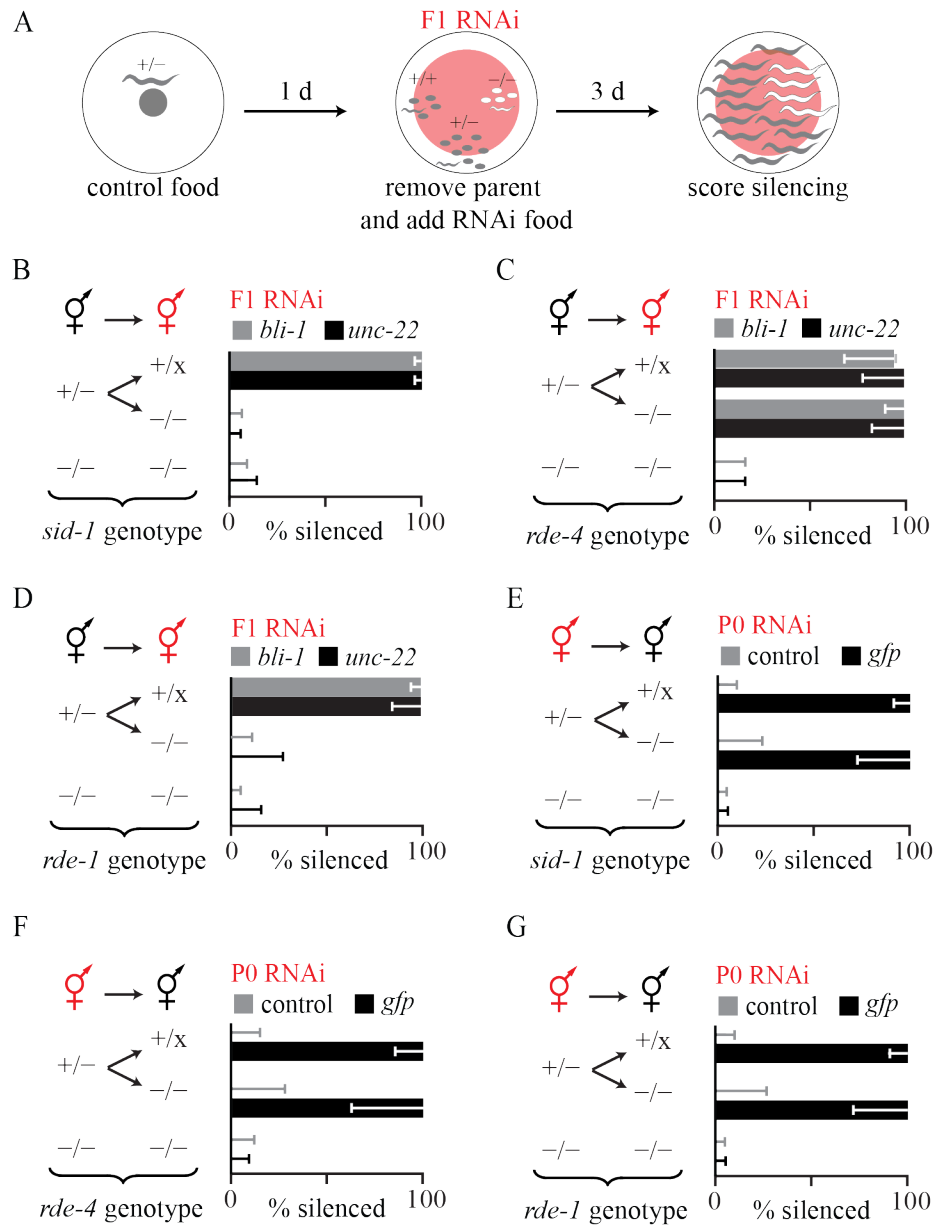


Fig. 2-8. Requirements for SID-1, RDE-4, and RDE-1 for gene silencing in progeny upon ingestion of dsRNA by parent or by progeny. (A) Schematic of F1 RNAi. Heterozygous parents (+/-, grey) were allowed to lay progeny on a small amount of control food. One day (1 d) later, the parents were removed and RNAi food (pink) was added to progeny (+/+ or +/-, grey and -/-, white). Three days later, the animals on RNAi food were scored for silencing. (B) Presence (+) of *sid-1* in parents was not sufficient for silencing (% silenced) of endogenous genes (hypodermal gene *bli-1*, grey bars; muscle gene *unc-22*, black bars) in *sid-1*(-) progeny when only progeny ingested matching dsRNA (F1 RNAi, red). (C and D) Presence (+) of *rde-4* (C) but not of *rde-1* (D) in parents was sufficient for silencing (% silenced) of endogenous genes (hypodermal gene *bli-1*, grey bars; muscle gene *unc-22*, black bars) in mutant progeny that lack the corresponding *rde* gene when

only progeny ingest dsRNA (F1 RNAi, red). (E) Presence (+) of *sid-1* in parents was sufficient for silencing (% silenced) of a single-copy *gfp* transgene (*Peft-3::gfp*) in progeny when parents ingested *gfp*-dsRNA. (F and G) Presence (+) of *rde-4* (F) and *rde-1* (G) in parents was sufficient for silencing (% silenced) of a single-copy *gfp* transgene (*Peft-3::gfp*) in the soma of progeny when only parents ingested *gfp*-dsRNA. Error bars indicate 95% CI (B-G); $x = +$ or $-$ (B-G); L4-staged animals were assayed ($n > 13$ (B), $n > 31$ (E), $n > 25$ (C), $n > 14$ (D), $n > 9$ (F), $n > 13$ (G)); and grey bars in (E)-(G) are as in Fig. 1. Entry of dsRNA into cytosol through SID-1, processing by RDE-4, and processing by RDE-1 can all occur in parents or during early development in progeny and be sufficient for silencing in progeny when parents ingest dsRNA. Parental RDE-4 can enable silencing in *rde-4(-)* progeny when progeny ingest dsRNA as larvae.

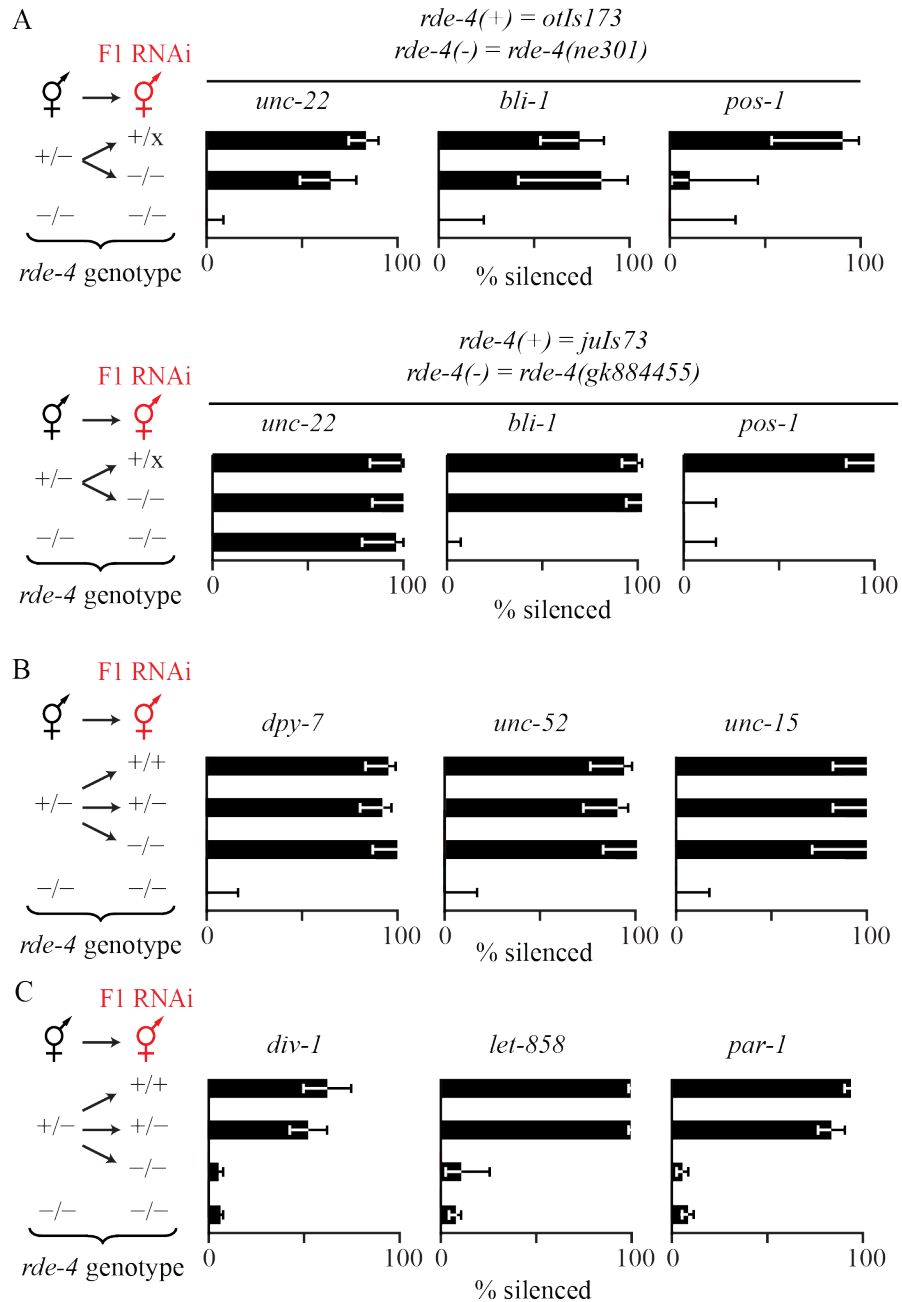


Fig. 2-9. Presence of *rde-4* in parents is sufficient for silencing somatic but not germline genes in *rde-4*(-) progeny when only progeny ingest dsRNA. (A) Presence (+) of *rde-4* in parents was sufficient for silencing (% silenced) of the somatic genes *unc-22* and *bli-1* but not the germline gene *pos-1* when only progeny (-/- or +/x, where x = + or -) ingested the corresponding dsRNA (F1 RNAi, red). Similar results were observed for both the strong *ne301* mutant allele (top, balanced with the fluorescent transgene *otIs173*) and the weak *gk884455* mutant allele (bottom, balanced with the fluorescent transgene *juIs73*) of *rde-4*. (B) Presence (+) of *rde-4* in parents was sufficient for silencing (% silenced) of the somatic genes *dpy-7*, *unc-52*, and *unc-15* in response to F1 RNAi. (C) Presence (+) of *rde-4* in parents was not sufficient for silencing (% silenced) of the germline genes *div-1*, *let-858*, and *par-1* in

response to F1 RNAi. Error bars indicate 95% CI and L4-staged animals were assayed ($n > 11$ (A), $n > 13$ (B), $n > 10$ (C)). Data generated by Ed Traver.

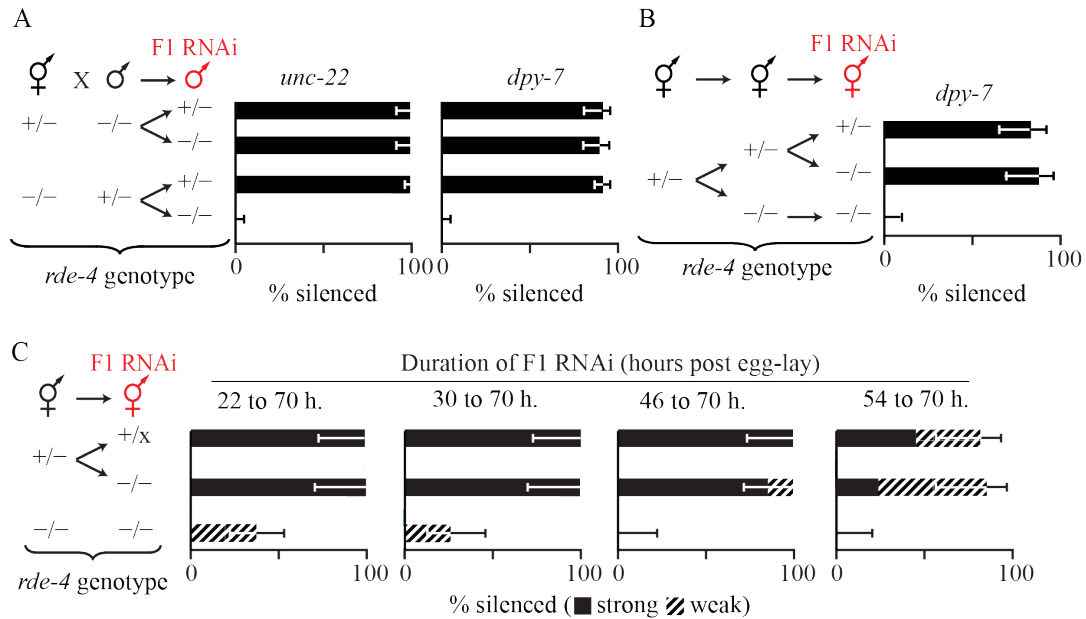


Fig. 2-10. Presence of *rde-4* in hermaphrodite parents is sufficient for silencing genes in *rde-4*(-) progeny that ingest dsRNA even when progeny ingest dsRNA as late as 54 hours post egg-lay. (A) Presence (+) of *rde-4* in hermaphrodite but not male parents was sufficient for silencing (% silenced) of the endogenous genes *unc-22* (left) and *dpy-7* (right) when only cross progeny ingested the corresponding dsRNA (F1 RNAi, red). (B) Presence (+) of *rde-4* in hermaphrodite grandparents was not sufficient for silencing (% silenced) of *dpy-7* when only mutant progeny of mutant progeny (i.e., mutant grandprogeny) ingested *dpy-7* dsRNA (F1 RNAi, red). (C) Presence (+) of *rde-4* in parents was sufficient for silencing (% silenced) of *unc-22* when progeny ingested *unc-22*-dsRNA even as late as 54 hours post egg-lay. Error bars indicate 95% CI and L4-staged animals were assayed ($n > 26$ (A), $n > 28$ (B), $n > 13$ (C)). Sufficient parental RDE-4 is present in progeny to enable silencing of genes expressed in somatic tissues – even when feeding RNAi is initiated beyond the fourth larval stage of progeny (*unc-22*). Data generated by Ed Traver.

If dsRNA is processed in a parental cell containing homologous mRNA – e.g., muscle cells for *unc-22*-dsRNA – processed derivatives of dsRNA could interact with mRNA to generate secondary small RNAs (Fig. 2-7). But, if *unc-22*-dsRNA is processed in cells without homologous mRNA, such as the germline, the dsRNA can only be processed to primary single-stranded siRNA (1° ss-siRNA, Fig. 2-7). To allow processing only in cells that lack matching mRNA and to allow subsequent inheritance of silencing signals to progeny, we expressed RDE-4 or RDE-1 under the control of a germline promoter (*Pmex-5*) and examined silencing in response to ingestion of *unc-22*-dsRNA. This promoter enabled expression within the germline and additionally within the intestine, but not within the muscle or hypodermis (Fig. 2-11). Expression of RDE-4 under the control of *Pmex-5* enabled silencing of *unc-22* in *rde-4(-)* progeny when either the parent with RDE-4 expression or progeny lacking RDE-4 expression ingested dsRNA, reflecting the persistence of parental RDE-4 in progeny (Fig. 2-12). However, such expression of RDE-1 under the control of *Pmex-5* enabled silencing in *rde-1(-)* progeny only when the parent with RDE-1 ingested dsRNA (Fig. 2-12). These results suggest that processing of ingested dsRNA by RDE-4 and subsequent processing by RDE-1 within cells that lack target mRNA in parents or within progeny during early development is sufficient for silencing in progeny. Thus, some primary RNAs, which could include long dsRNA, 1° ds-siRNA, and 1° ss-siRNA, are inherited from parents to progeny.

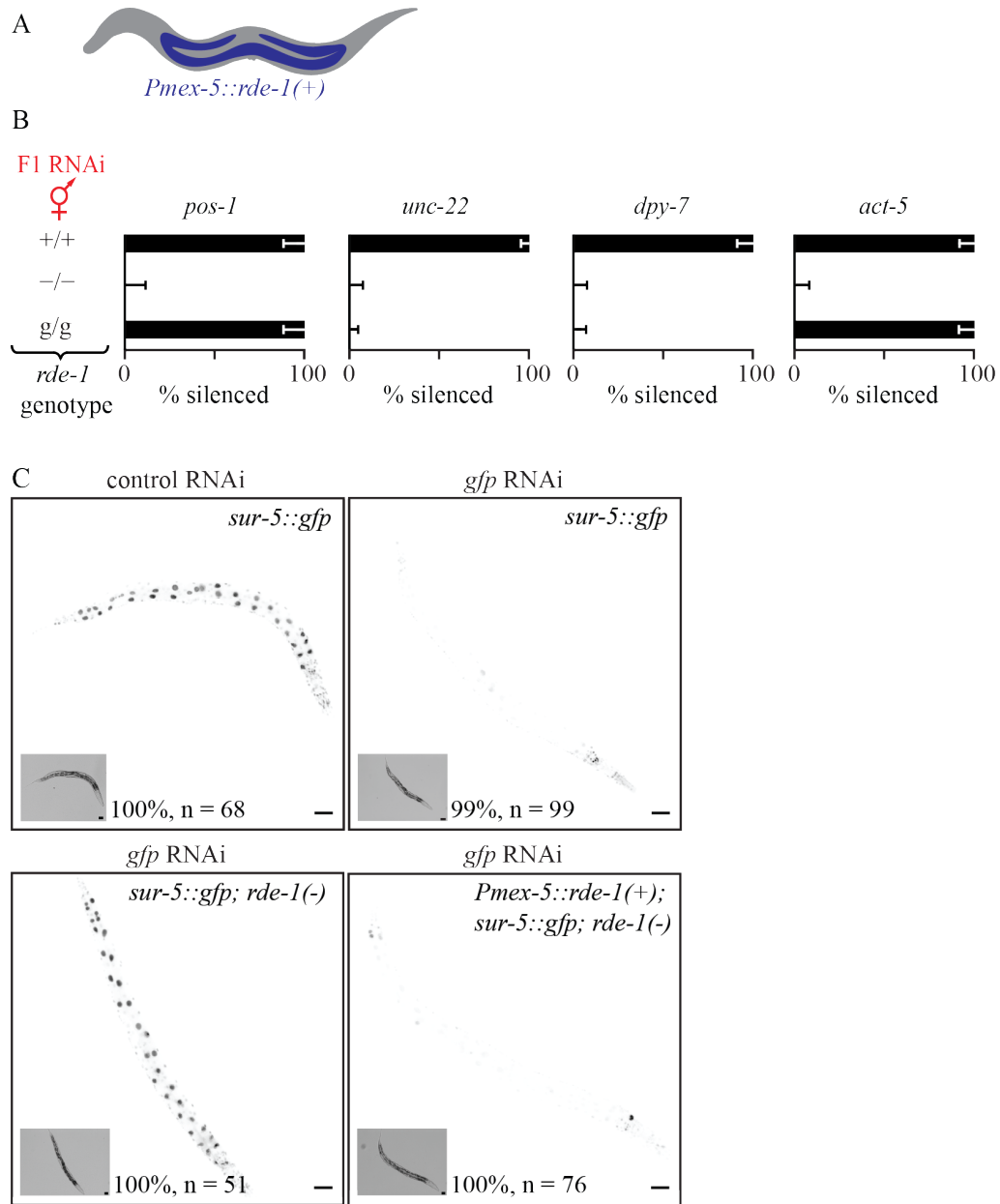


Fig. 2-11. Expression of RDE-1 under the control of the *mex-5* promoter enables silencing within the germline and intestinal cells but not within hypodermal or muscle cells. (A) Schematic of worm showing the expression of *rde-1* restricted to the germline (blue) with a promoter reported to be specific to the germline (*Pmex-5::rde-1(+)*). *dpy-7* and *act-5* data generated by Pravrutha Raman. (B) Expression of *rde-1* under the control of *Pmex-5* (g) was sufficient for silencing (% silenced) the germline gene *pos-1* and the intestinal gene *act-5* but not the muscle gene *unc-22* or the hypodermal gene *dpy-7* when animals ingested the corresponding dsRNA (F1 RNAi, red). Error bars indicate 95% CI and L4-staged animals were assayed (n > 42). (C) Expression of *rde-1* under the control of *Pmex-5* (g) also supports silencing of *gfp* expression within the intestine. Silencing of *gfp* by F1 RNAi in animals that express

sur-5::gfp in a wild-type, *rde-1(-)*, or *rde-1(-); Pmex-5::rde-1(+)* background was scored and representative worms were imaged (% indicates animals with similar phenotype and n indicates number of L4 animals scored). Insets are bright-field images and scale bar = 50 μ m. Silencing of *gfp* was not observed upon F1 RNAi in \sim 25% of progeny from *sur-5::gfp; rde-1(-); Pmex-5::rde-1(+)* parents (n > 25 F1s each from 5 P0 animals; total n = 206 L4 animals), consistent with lack of silencing in *sur-5::gfp; rde-1(-)* progeny and with lack of rescue from *rde-1(+)* expression within the parental germline. These results suggest that our strain, with expression of *rde-1(+)* under the control of a *mex-5* promoter, supports gene silencing by feeding RNAi in germline and intestinal cells but not in muscle or hypodermal cells.

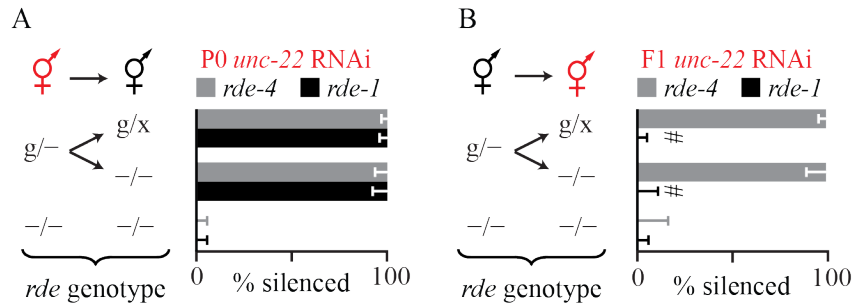


Fig. 2-12. Processing of ingested dsRNA in parents or in embryos is sufficient for silencing in progeny. (A and B) Silencing of *unc-22* in progeny of animals with *rde-4* (grey bars) or *rde-1* (black bars) expressed within the germline and the intestine under the *mex-5* promoter (*Pmex-5::rde(+)* = g). Progeny genotypes (-/- or g/x, where x = + or g) and type of feeding RNAi (P0 *unc-22* RNAi or F1 *unc-22* RNAi) are indicated. Hashtags = much weaker silencing in all animals. Error bars indicate 95% CI and L4-staged animals were assayed (n > 56 (A) and n > 42 (B)).

When radioactively-labeled dsRNA is injected into the germline, a large fraction of the dsRNA remains as high molecular weight material in progeny (77, 78), consistent with substantial delivery of long dsRNA into progeny. When *unc-22*-dsRNA was similarly delivered directly into the parental germline, and thus into the cytosol of cells in the embryo, potent silencing occurred in early-born progeny in

wild-type and in *sid-1(-)* animals (Fig. 2-7 D), suggesting that sufficient dsRNA was delivered into all cells in early progeny. In contrast, later-born progeny, which are expected to receive smaller doses of dsRNA because of dilution within the germline (73), required SID-1 for efficient silencing (Fig. 2-7 D). This need for SID-1-dependent spread of dsRNA between cells for efficient silencing in later progeny is consistent with forms of dsRNA (long dsRNA and/or 1^o ds-siRNA) being inherited from the injected parents to progeny.

Taken together, these results suggest that intracellular primary small RNAs – including forms of dsRNA – can be inherited from parent to progeny and explain the surprising observation that when animals that lack DNA of matching sequence ingest dsRNA, they can still transmit a silencing signal to progeny.

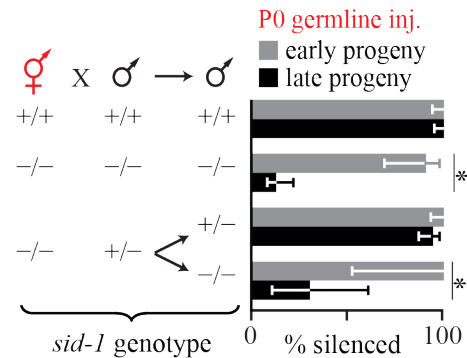


Fig. 2-13. Inherited dsRNA spreads between cells in the embryo to cause silencing. Presence (+) of *sid-1* was necessary in late progeny (laid 72 hours post injection, black) for ~100% silencing of *unc-22* when *unc-22*-dsRNA was injected into the germline of hermaphrodite parents (red, P0 germline inj.). Asterisks indicate $P < 0.05$ (Student's *t*-test); error bars indicate 95% CI; and L4-staged animals were assayed ($n > 22$).

2.4.4 Extracellular dsRNA can cause silencing in progeny without SID-1-dependent entry in parents

Ingested dsRNA can cross the intestine into the fluid-filled body cavity that surrounds all tissues including the germline without SID-1-dependent entry into the cytosol of intestinal cells (51, 57, 79). To determine where subsequent entry into the cytosol in parents is required for silencing in progeny, we delivered dsRNA into the extracellular space by injecting into the body cavity beyond the bend of the posterior gonad arm (Fig. 2-14 *A* and Fig. 2-15 *A*). Such injection resulted in the immediate spread of the injected material by diffusion throughout the entire body cavity as evidenced by injection of fluorescently labeled dextran (Fig. 2-15 *B*). Entry of dsRNA from the body cavity into the cytosol of a cell is expected to occur only in cells that have the dsRNA importer SID-1 (38). Consistent with results from feeding RNAi, presence of SID-1 in parents was sufficient for silencing in progeny in response to dsRNA injected into the body cavity (Fig. 2-15 *C* and *D*). However, when SID-1 was restricted to either sperm (*sid-1(+)* male) or to all cells except sperm (*sid-1(+)* hermaphrodite), injection of dsRNA targeting the muscle gene *unc-22* (*unc-22*-dsRNA) into the body cavity of hermaphrodites enabled *unc-22* silencing in cross progeny (Fig. 2-14 *B* and Fig. 2-15 *E*), as was reported earlier (38). Taken together with the dynamics of silencing by ingested dsRNA (Fig. 2-1 and Fig. 2-6), these results suggest that injected extracellular dsRNA can be transported to the next generation through oocytes, presumably within intracellular vesicles, from which the entry of dsRNA into the cytosol through SID-1 occurs in progeny. Consistently, any paternal SID-1 protein or mRNA present in sperm from heterozygous males was not

sufficient for silencing, and zygotic expression of SID-1 in progeny was required for silencing (Fig. 2-14 *B*, compare fifth bar with sixth bar). Similarly, in the case of dsRNA transported from neurons (33), the neuronal dsRNA from animals that lack SID-1 could cause silencing in cross progeny when mated with wild-type animals (Fig. 2-14 *C*). Thus, these results suggest that extracellular dsRNA does not need to enter the cytosol in parental tissues to cause silencing in progeny – cytosolic entry within the progeny is sufficient.

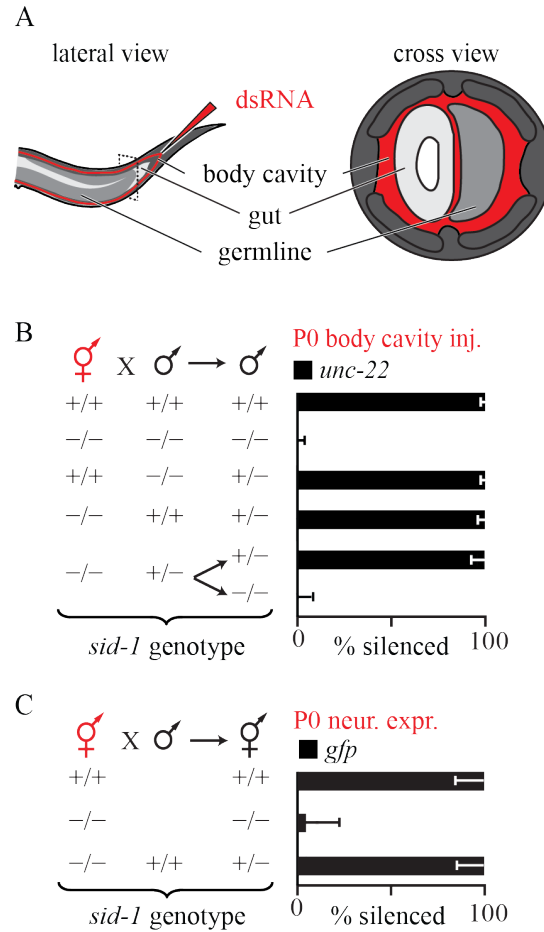


Fig. 2-14. Extracellular dsRNA does not need to enter the cytosol of any cell in parents to cause silencing in progeny. (A) Schematic showing injection of dsRNA into the body cavity. (B) Presence (+) of *sid-1* in progeny was sufficient for silencing of an endogenous gene (*unc-22*) in the muscles of progeny when dsRNA matching the gene (*unc-22*-dsRNA, red) was injected into the body cavity of hermaphrodite parents. Also see Fig. S9. (C) Presence (+) of *sid-1* in hermaphrodite parents was not required for silencing of a single-copy *gfp* transgene (*Pmex-5::gfp*) in the germline of progeny when *gfp*-dsRNA was expressed within parental neurons (P0 neur. expr., red) of hermaphrodite parents. Data generated by Sindhuja Devanapally. Error bars indicate 95% CI (B and C) and L4-staged animals were assayed (n > 49 (B), n > 38 (C)).

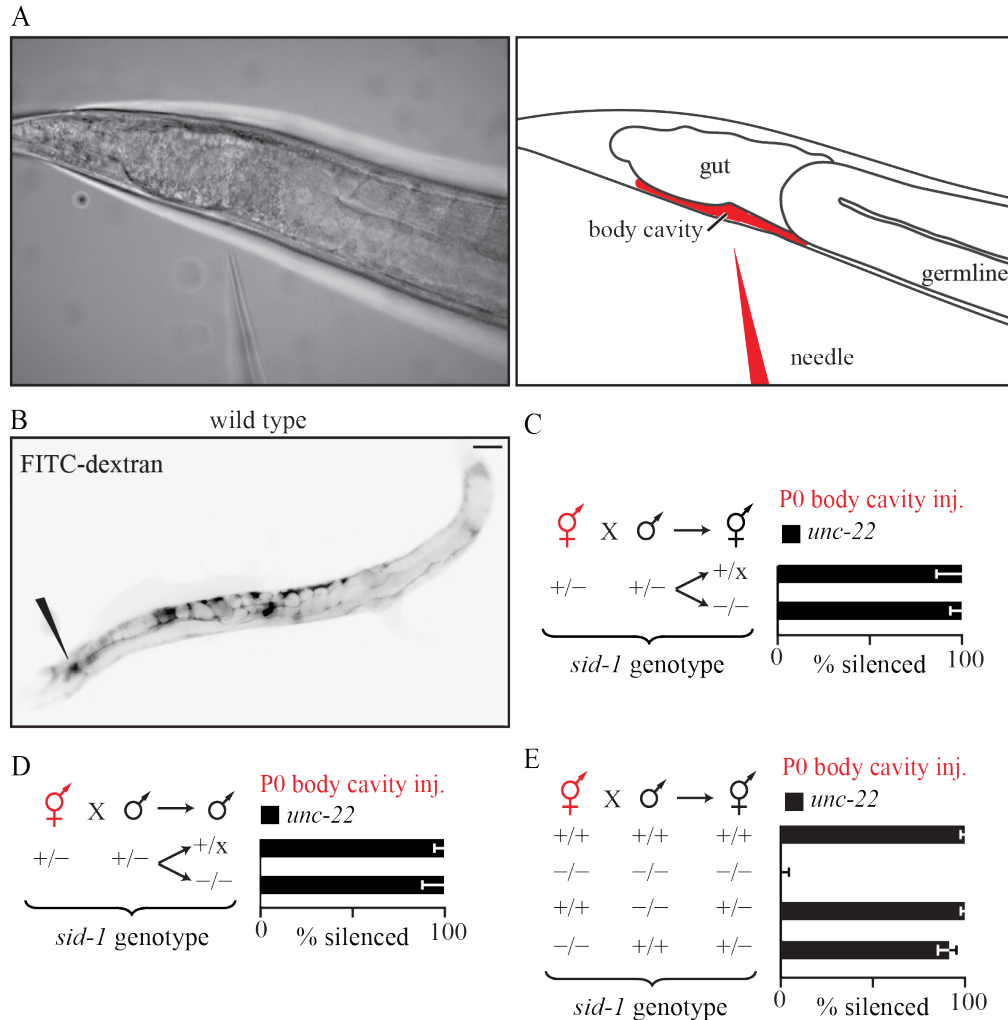


Fig. 2-15. Presence of SID-1 in parents or progeny is sufficient for silencing in progeny when dsRNA is injected into the body cavity of parents. (A) Injection of dsRNA into the body cavity (Fig. 3-6) was performed by inserting a microinjection needle containing dsRNA (needle) past the bend of the posterior gonad arm. Representative image of set up (*left*) and schematic (*right*) for injection are shown. (B) Representative image of fluorescence (black) in a wild-type adult animal from fluorescein-labeled 10 kDa dextran injected into the body cavity (black needle indicates site of injection). (C and D) Presence (+) of *sid-1* in parents was sufficient for silencing (% silenced) of an endogenous gene (*unc-22*) in the muscles of hermaphrodite (C) and male (D) progeny when dsRNA matching the gene (*unc-22*-dsRNA, red) was injected into the body cavity of hermaphrodite parents. (E) Injections and crosses were performed as described in Fig. 3, A and B and hermaphrodite cross progeny were assayed for silencing. Scale bar = 50 μ m (B), error bars indicate 95% CI (C-E); x = + or - (D-E); and L4-staged animals were assayed ($n > 31$ (C), $n > 35$ (D), $n > 139$ (E)).

2.5 Discussion

In this chapter, genetic analyses were used to propose a model where extracellular dsRNA itself is transported to progeny without entering the cytosol of any cell in parents and can enter the cytosol in progeny (Fig. 2-16). This model suggests that extracellular dsRNA transits through the germline restricted from the cytosol, potentially within a membrane-bound vesicles (Fig. 2-16). Extracellular dsRNA is likely transmitted through oocytes and not sperm (Fig. 2-1 and Fig. 2-16) and both long dsRNA and processed short dsRNA species reach progeny (Fig. 2-12, 2-13 and Fig. 2-14).

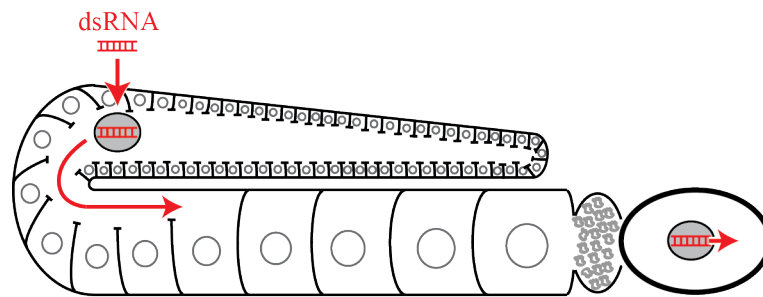


Fig. 2-16. Extracellular dsRNA is transported to progeny through oocytes without entering the cytosol in parents. Red arrows indicate the path of dsRNA transport through the germline to progeny.

2.5.1 Spread of cytosolic dsRNA species between cells in the embryo

dsRNA introduced into the rachis of the germline and thus into the 1-cell embryo was only able to initiate silencing in muscle cells in late-born progeny containing SID-1 (Fig. 2-7 C), suggesting that the spread of a dsRNA species between cells was necessary for silencing. This result suggests that as the 1-cell

embryo develops, there are mechanisms to unequally segregate cytosolic dsRNA into some cell descendants and not others, requiring the spread of dsRNA between cells to silence genes in cells that did not receive dsRNA upon cell division. Alternatively, genes required to process long dsRNA into short primary ds-siRNA (e.g., *rde-4*) may only be present in some cell descendants requiring the initial processing of long dsRNA in those cells and the spread of primary ds-siRNA to cells without processing genes. Consistently, transgenic dsRNA expressed in early gut cells can spread through SID-1 in ~60-cell staged embryo to silence some gut cells (80).

2.5.2 *mex-5* promoter is not restricted to the germline

The promoter for *mex-5* is often used in *C. elegans* transgenics to express genes of interest in the germline. However, we observed that driving the expression of *rde-1* under the *mex-5* promoter (*Pmex-5::rde-1*) enabled silencing of two different genes in the gut (Fig. 2-11), suggesting that in addition to the germline, *Pmex-5* also expresses in the gut. This is consistent with transcriptional reporters of *mex-5* (81).

2.5.3 Chromatin marks are not necessary for silencing in progeny

In *C. elegans*, there are many maternal mRNAs, piRNAs, and endogenous siRNAs deposited from within the cytosol of the germline through oocytes to the developing embryo and some RNAs have also been detected in sperm that also reach the zygote (82). Our data suggest that extracellular RNA can be transmitted through oocytes and not sperm to silence genes in progeny (Fig. 2-1, Fig. 2-14, and Fig. 2-15).

Our work also showed that the target DNA locus is not necessary in parents for silencing in progeny (Fig. 2-6), suggesting that the production of secondary amplified RNA signals using mRNA and placement of chromatin marks on DNA is

not necessary for silencing in progeny. We also suggest that RNA present in circulation can itself be inherited without entering the cytosol of the germline (Fig. 2-16).

Chapter 3: Fluorescently-labeled extracellular RNA accumulates in proximal oocytes by vitellogenin-associated endocytosis

3.1 Preface

Some of the work presented in this chapter was published as: Marré JA, Traver EC, and Jose AM (2016) Extracellular dsRNA is transported from one generation to the next in *C. elegans*. *Proceedings of the National Academy of Sciences USA* 113(44):12496-501.

All data in this chapter was generated by Julia Marré.

Some worm strains were obtained from the *Caenorhabditis elegans* Genetic stock Center, the Hunter laboratory (Harvard University), and the Seydoux laboratory (Johns Hopkins University). The Hamza laboratory (University of Maryland) provided some of the bacteria that express dsRNA. The Andrews laboratory (University of Maryland) trained me and allowed me to use their confocal microscope.

3.2 Introduction

Our genetic analyses in Chapter 2 suggested a model where extracellular dsRNA is transported through oocytes to progeny without entering the cytosol of the germline (Fig. 2-14). Here, we used cell biological approaches to visualize extracellular dsRNA while it was being transported from the parental germline to

progeny embryos using fluorescently labeled dsRNA. Using genetic mutant backgrounds, we defined some of the mechanisms of dsRNA uptake into oocytes.

Here, we visualized how extracellular dsRNA accumulate within oocytes and reach progeny. Entry into oocytes was dependent upon the vitellogenin endocytosis pathway and independent of the dsRNA importer SID-1. Silencing in the embryo by inherited RNA was observed when SID-1 was present.

3.3 Materials and methods

3.3.1 Strains, transgenesis, and oligonucleotides

All strains used are listed in Table 3-1 and all oligonucleotides used are listed in Table 3-2. Strains were cultured and maintained as described in (70).

Table 3-1. Strains used.

Strain	Genotype
AMJ795	<i>rme-2(b1008) IV; pwIs23 [Pvit-2::vit-2::gfp]</i>
DH1390	<i>rme-2(b1008) IV</i>
HC196	<i>sid-1(qt9)</i>
N2	wild type
RT130	<i>pwIs23 [Pvit-2::vit-2::gfp]</i>
TX691	<i>unc-119(ed3) III; telIs46</i>

Table 3-2 Oligonucleotides used (5' to 3', IDT).

Oligonucleotide	Sequence
P1 (<i>rme-2(b1008)</i> genotype fwd)	acaaccactacgccagaagg
P2 (<i>rme-2(b1008)</i> genotype rev)	actcggcgacagaacattcc
P3 (<i>gfp</i> sense RNA)	ugguccuucuugaguuuguaacagcugcugggauuacaca uggcauggau
P4 (5' Atto 565 labeled <i>gfp</i> antisense ssRNA)	5'Atto 565-auccaugccauguguaauccagcagcuguu acaaacucaagaaggacca

To place *vit-2::gfp* in an *rme-2(-)* background: RT130 (*Pvit-2::vit-2::gfp*) hermaphrodites were mated with N2 males and cross progeny males were then mated with DH1390 (*rme-2(b1008)*) hermaphrodites. F2 progeny were then genotyped using primers P1 and P2 to generate AMJ795.

3.3.2 P0 feeding RNAi

Inheritance assay in response to P0 RNAi: RNAi bacteria was grown in LB-carbenicillin overnight and 100 µl was seeded on RNAi plates (NG agar plate supplemented with 1mM IPTG (Omega) and 25 µg/ml carbenicillin (MP Biochemicals)). Seeded RNAi plates were incubated at room temperature for 1-2 days before L4-staged worms were added. The plates were then incubated at 20°C for one day. RNAi bacteria were then removed by a 4x wash.

4x wash: The RNAi fed worms were suspended in 1 ml of M9 buffer in a 1.5 ml microcentrifuge (VWR) tube and spun at 8,000 rpm for 30 seconds. After

removing 800 μ l of the old buffer, an equal volume of fresh M9 buffer was added. This washing was repeated four times and the final 200 μ l of M9 buffer with worms was placed on plates seeded with OP50 and incubated for one hour at room temperature before each worm was moved to a fresh plate seeded with OP50.

The earliest L4-staged progeny were scored (~20 per worm) for inherited gene silencing by placing worms in 3mM levamisole and observing the twitching phenotype within 1 minute (typically two to three days later except in the case of slow growing *rme-2(-)* strain, which was scored five days later).

3.3.3 Injection of dsRNA, dextran, and Atto 565 dye

Annealing Atto 565-labeled *gfp*-dsRNA: ssRNA oligos P3 and P4 were resuspended in nuclease free 1X TE (IDT) to ~1635 ng/ μ l. Equal volumes of each ssRNA were mixed in nuclease free duplex buffer (IDT) to a final concentration of ~490 ng/ μ l of each ssRNA. This mixture was heated to 95°C and cooled at 1 min/degree to 10°C, resulting in ~980 ng/ μ l of dsRNA. P3, P4, and the annealed dsRNA were run in a 12% denaturing polyacrylamide gel. The gel was first imaged with the Typhoon Trio Variable Mode Imager (GE Healthcare) using a 532 nm laser and then stained with ethidium bromide (amresco) followed by imaging with the Molecular Imager Gel Doc XR (Bio-Rad) using ultraviolet light.

Injection: Adult animals (24 hours post L4-stage) were injected with ~325 - 980 ng/ μ l *gfp*-dsRNA (Fig. 3-2 E, Fig. 3-3, Fig. 3-5 A, Fig. 3-6 A, Fig. 3-7 B, Fig. 3-8), 0.25 mg/mL fluorescein-labeled 10kDa dextran (Life Technologies, D-1821) (Fig. 3-5 B, Fig. 3-6 B, Fig. 3-7 C), or 18.3 ng/ μ l Atto 565 dye (Sigma, 75784) (Fig. 3-4 B) into the body cavity past the bend of the posterior arm of the gonad.

3.3.4 Fluorescence imaging

Embryos from adults injected with fluorescent *gfp*-dsRNA: Embryos laid by adults 3 to 5 hours post injection on agar plates were individually imaged at fixed magnification on an AZ100 microscope (Nikon) with a Cool SNAP HQ² camera (Photometrics). A C-HGFI Intensilight Hg Illuminator was used to excite GFP (filter cube: 450-490 nm excitation, 495 dichroic, and 500-550 nm emission) or DsRed/Atto 565 (filter cube: 530-560 nm excitation, 570 dichroic, and 590-650 nm emission). Exposure times were scaled for control embryos laid by uninjected adults to just under saturation for each genetic background and then embryos laid by injected adults were imaged using the same exposure time. Corresponding bright-field images were taken using auto-exposure. Images were adjusted for display using ImageJ (NIH).

Adults injected with fluorescent *gfp*-dsRNA, dextran, and Atto 565 dye: 2.5 to 3 hours (Fig. 3-2, Fig. 3-4, Fig. 3-5, Fig. 3-6, Fig. 3-7, and Fig. 3-8) or 7 minutes (Fig. 3-3) post injection, adults were placed in 3mM tetramisole hydrochloride (Sigma) and imaged using the Eclipse Ti Spinning Disk Confocal (Nikon) with the 60X objective lens. Atto 565 was excited using a 561 nm laser and fluorescence was collected through a 415-475 nm and 580-650 nm emission filter. GFP was excited using a 488 nm laser and fluorescence was collected through a 500-550 nm emission filter. Images were adjusted for display using ImageJ (NIH).

Comparison of brightness level in Atto 565 dye and Atto 565-labeled dsRNA (Fig. 3-4 A): 0.5 μ l of Atto 565 dye or Atto 565-labeled dsRNA used for injections was placed on parafilm and imaged on an AZ100 microscope (Nikon) with a Cool SNAP HQ² camera (Photometrics). A C-HGFI Intensilight Hg Illuminator was used

to excite DsRed/Atto 565 (filter cube: 530-560 nm excitation, 570 dichroic, and 590-650 nm emission).

3.4 Results

3.4.1 Extracellular dsRNA accumulates in proximal oocytes and persists in embryos to silence a gene of matching sequence

Genetic analyses collectively suggest that extracellular dsRNA can reach progeny to silence a matching gene (Chapter 2). To track extracellular dsRNA between generations, we labeled 50-bp *gfp*-dsRNA with a fluorophore at a 5' end (Fig. 3-1), injected the fluorescently-labeled *gfp*-dsRNA into the body cavity of transgenic worms with embryonic expression of GFP, and imaged the germline (Fig. 3-2 *A*) of injected animals as well as embryos (Fig. 3-2 *B* and *C*) laid by injected adults (Fig. 3-2 *D*). Fluorescently-labeled dsRNA injected into the body cavity accumulated as puncta within oocytes in adult animals (Fig. 3-2 *E*). These intracellular puncta could be detected ~7 minutes after injection (Fig. 3-3). Levels of fluorescent dsRNA decreased from proximal to distal oocytes such that the oocyte located proximal to the spermatheca (-1 oocyte) had the highest fluorescence and most of the fluorescence within oocytes was limited to the two most proximal oocytes (Fig. 3-2 *E*). Fluorescent RNA could also be detected in embryos (Fig. 3-2 *F-I*), suggesting that extracellular dsRNA imported into oocytes persists in embryos. Furthermore, all embryos with fluorescent dsRNA also showed silencing of *gfp* expression (compare Fig. 3-2 *C* with Fig. 3-2 *G*). Together, these results demonstrate

the entry of extracellular dsRNA into oocytes and their persistence in embryos to silence genes of matching sequence.

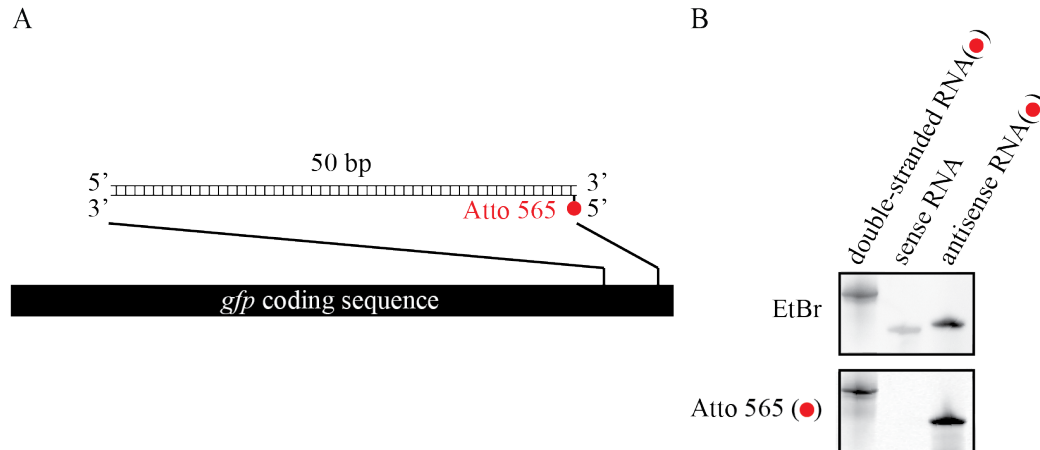


Fig. 3-1. Annealing sense RNA and Atto 565-labeled antisense RNA generates fluorescent dsRNA. (A) Schematic of fluorescent *gfp*-dsRNA. The Atto 565 label is attached to the 5' end of the antisense strand of dsRNA. (B) RNA was run in a 12% polyacrylamide gel and imaged for fluorescence (*bottom*, Atto 565) and stained with ethidium bromide (*top*, EtBr).

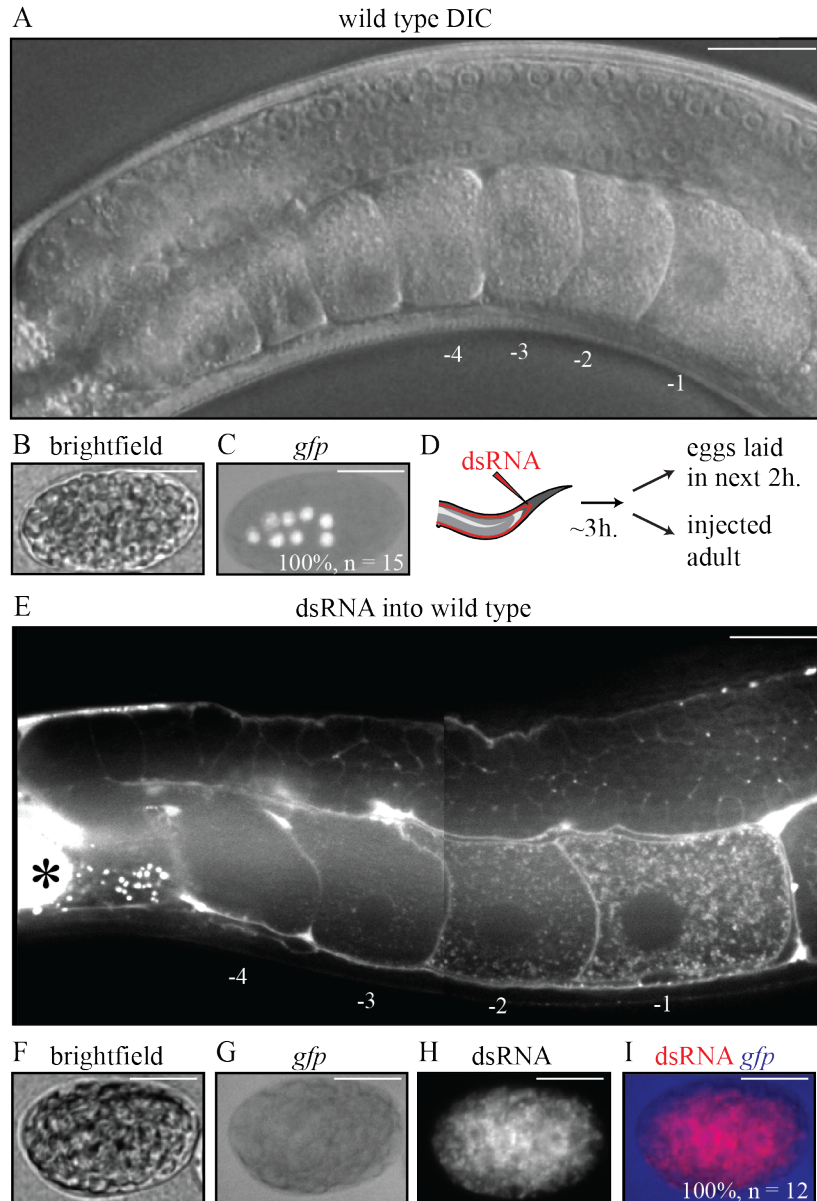


Fig. 3-2. Extracellular dsRNA accumulates in proximal oocytes and subsequently within embryos where it can silence genes of matching sequence. (A-C) Images of adult germline showing proximal oocytes (-1 through -4 with respect to the spermatheca) (A) and embryos (B) expressing GFP in intestinal cells (*Pend-1::gfp*) (C). (D) Strategy to visualize silencing by fluorescently labeled *gfp*-dsRNA. (E) In injected wild-type animals, dsRNA concentrated in proximal oocytes (-1 and -2). Slices from two Z-series were spliced. Asterisk indicates brightly fluorescent intestinal cell. (F-I) Wild-type embryos inherited the *gfp*-dsRNA and silencing of *Pend-1::gfp* occurred. Scale bars, 20 μ m. Multiple adults (n = 4 in (E)) and embryos (n = 15 in (B) and (C), and n = 12 in (F-I)) were imaged for each experiment.

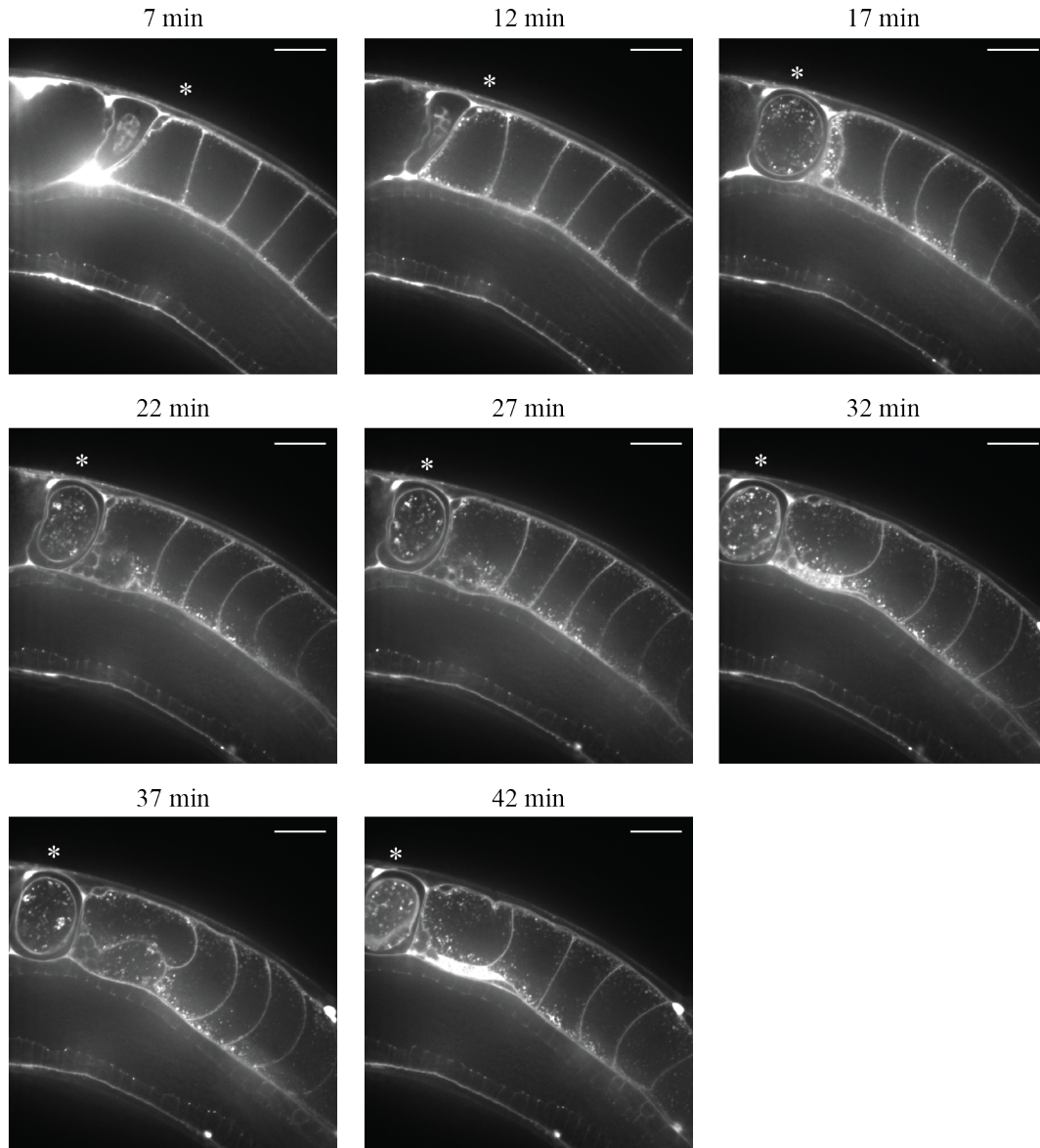


Fig. 3-3. Accumulation of dsRNA in proximal oocytes can be detected as early as 7 minutes post injection. Fluorescent dsRNA was injected into the body cavity and the same Z-stack was imaged every 5 minutes beginning with 7 minutes post injection. Asterisk indicates the location of the -1 oocyte at 7 minute post injection and as the oocyte progresses through fertilization to become an embryo. Scale bars, 20 μ m.

3.4.2 Atto 565 dye does not enter oocytes

Atto 565-labeled dsRNA appeared to accumulate as puncta in oocytes when injected into the body cavity of adult animals (Fig. 3-2 *E*). However, when Atto 565 dye alone was injected into the body cavity of adult animals in a concentration at comparable brightness level to Atto 565-labeled dsRNA (Fig. 3-4 *A*), no puncta were detectable in oocytes (Fig. 3-4 *B*, *top*). It is unlikely that Atto 565 dye has been eliminated from the body cavity thus preventing entry into oocytes because Atto 565 dye could be detected in the coelomocytes or scavenger cells in the body cavity (Fig. 3-4 *B*, *bottom*), however, the rate of renal clearance may differ for the Atto 565 label alone vs. Atto 565-labeled dsRNA. Coelomocytes can endocytose molecules from the body cavity as fast as 5 min after molecules are introduced into the body cavity (Texas Red-conjugated BSA injected into the body cavity) (83), which is at about the same rate we observed fluorescent dsRNA accumulation in oocytes. These results suggest that the entry of Atto 565-labeled dsRNA into oocytes is specific to dsRNA and not the Atto 565 label.

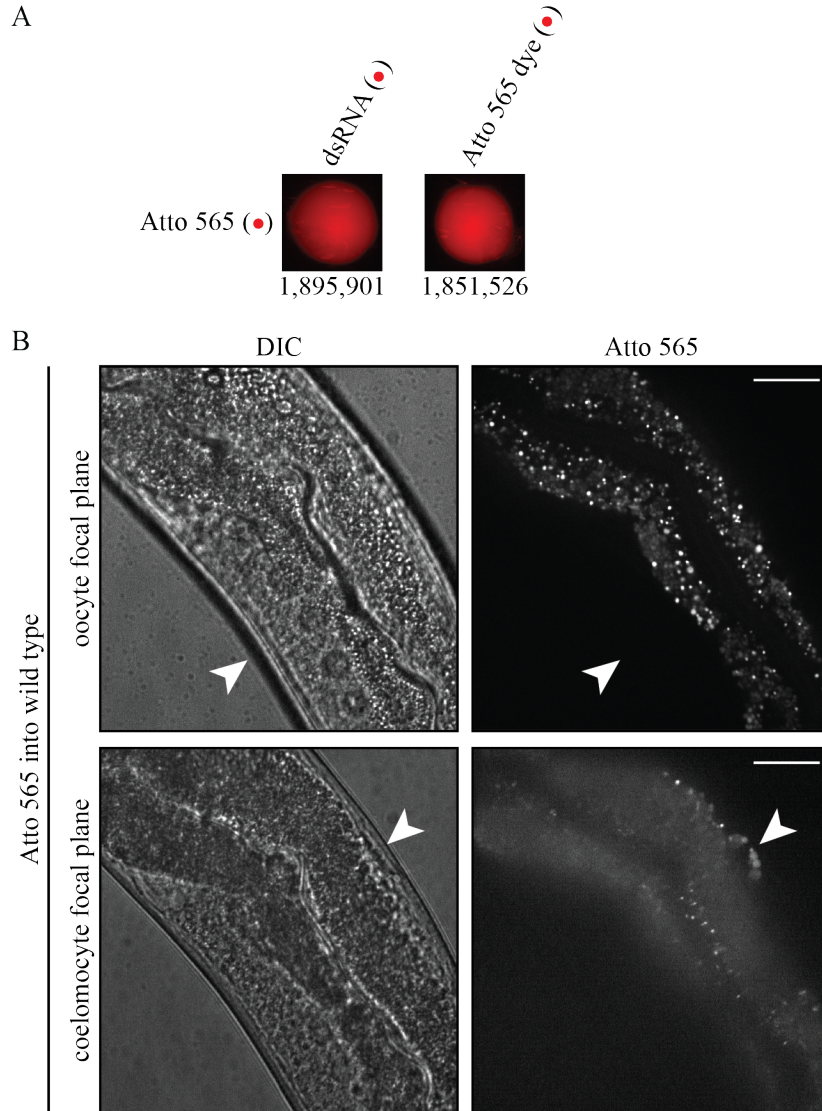


Fig. 3-4. Atto 565 dye alone does not accumulate in oocytes. (A) Atto 565-labeled dsRNA and Atto 565 dye injected into the body cavity are at similar brightness levels. (B) Atto 565 dye injected into the body cavity of wild type animals accumulates in coelomocytes (*bottom*) but not oocytes (*top*). Arrowheads indicate location of oocytes (*top*) and coelomocyte (*bottom*). Scale bars, 20 μ m. n = 4 adults injected and imaged.

3.4.3 Extracellular dsRNA enters oocytes along with vitellogenin

The accumulation of dsRNA in two proximal oocytes (Fig. 3-2 *E*) is reminiscent of the accumulation of yolk proteins (vitellogenins), which also similarly accumulate in proximal oocytes (66). To directly compare dsRNA import with vitellogenin import, we injected fluorescently-labeled dsRNA into the body cavity of animals that express a GFP-tagged vitellogenin, VIT-2::GFP (Fig. 3-5 *A, left*). Both dsRNA and VIT-2::GFP accumulated as puncta within the proximal oocytes (Fig. 3-5 *A, left*), consistent with their import being mediated by the same process. The import of vitellogenin into *C. elegans* oocytes occurs through endocytosis and requires the receptor RME-2, a member of the low-density lipoprotein receptor superfamily (66). In animals that lack RME-2, both GFP-tagged vitellogenin and fluorescently-labeled dsRNA accumulated extracellularly in the body cavity without import into oocytes (Fig. 3-5 *A, right*). The co-accumulation of dsRNA with vitellogenin is likely to be through non-specific import of material from the body cavity during the uptake of the ~0.5 μm -sized vitellogenin granules (84) because fluorescently labeled dextran (10 kDa) also accumulated in punctate structures within oocytes (Fig. 3-5 *B*). Consistent with the results from injected dsRNA, RME-2 was required for silencing in progeny when parents ingested dsRNA (Fig. 3-5 *C*). Together, these results suggest that the import of dsRNA into oocytes occurs through the same pathway that imports vitellogenin in *C. elegans* - RME-2-mediated endocytosis.

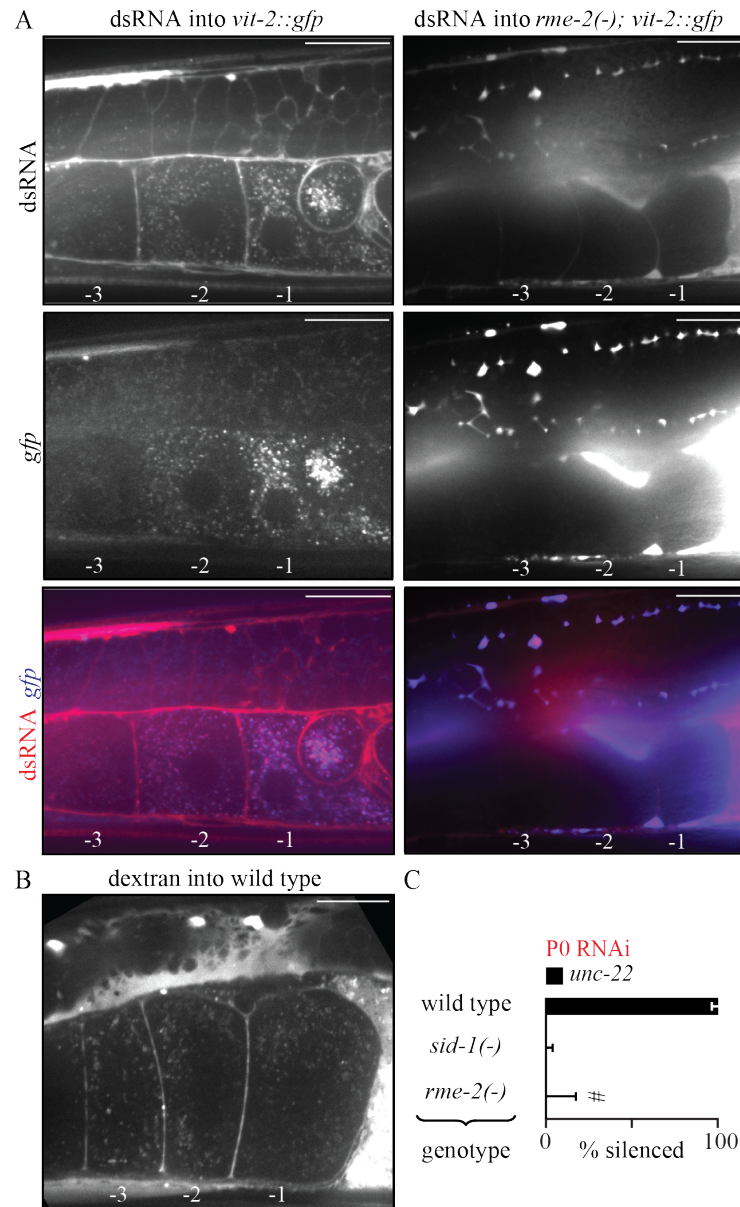


Fig. 3-5. Import of dsRNA into oocytes relies on RME-2-mediated endocytosis. (A) Import of dsRNA and of Vitellogenin into oocytes both require RME-2. Fluorescently labeled dsRNA (*top*) and Vitellogenin-2::GFP (*middle, gfp*) accumulate similarly in proximal oocytes (*bottom, merge*) in wild-type (*left*) but not in *rme-2(-)* (*right*) animals. (B) Fluorescent dextran from the body cavity also accumulates in punctate structures within oocytes. Scale bars, 20 μ m; and proximal oocytes are numbered as in Fig. 4. Multiple adults (n = 4 in (A, left), n = 5 in (A, right), n = 3 in (B)) were imaged for each experiment. (C) Ingestion of dsRNA by animals that lack *rme-2* (*rme-2(-)*) does not result in detectable silencing in progeny. Hashtag = much weaker silencing in 2 of 23 animals. Error bars and grey bars are as in Fig. 1, and n > 22 L4-staged animals. Note: A large circular enclosure of cytoplasmic material was observed, typically during ovulation, in 8/29 of the -1 oocytes imaged across multiple

genotypes (e.g., *A*, left). This structure is either a normal feature of ovulation that is seen when the extracellular space is fluorescently labeled or is the result of constrained ovulation under a coverslip. This circular enclosure can also be seen over time in Fig. 3-3.

3.4.4 dsRNA, dextran, and vitellogenin accumulate in coelomocytes

Molecules present in the body cavity in *C. elegans* can be subject to endocytosis by scavenger cells called coelomocytes. Consistently, fluorescently-labeled dsRNA and FITC-labeled dextran injected into the body cavity accumulated in coelomocytes (Fig. 3-6). Vitellogenin-2::GFP also accumulated in coelomocytes consistent with previous observations (66).

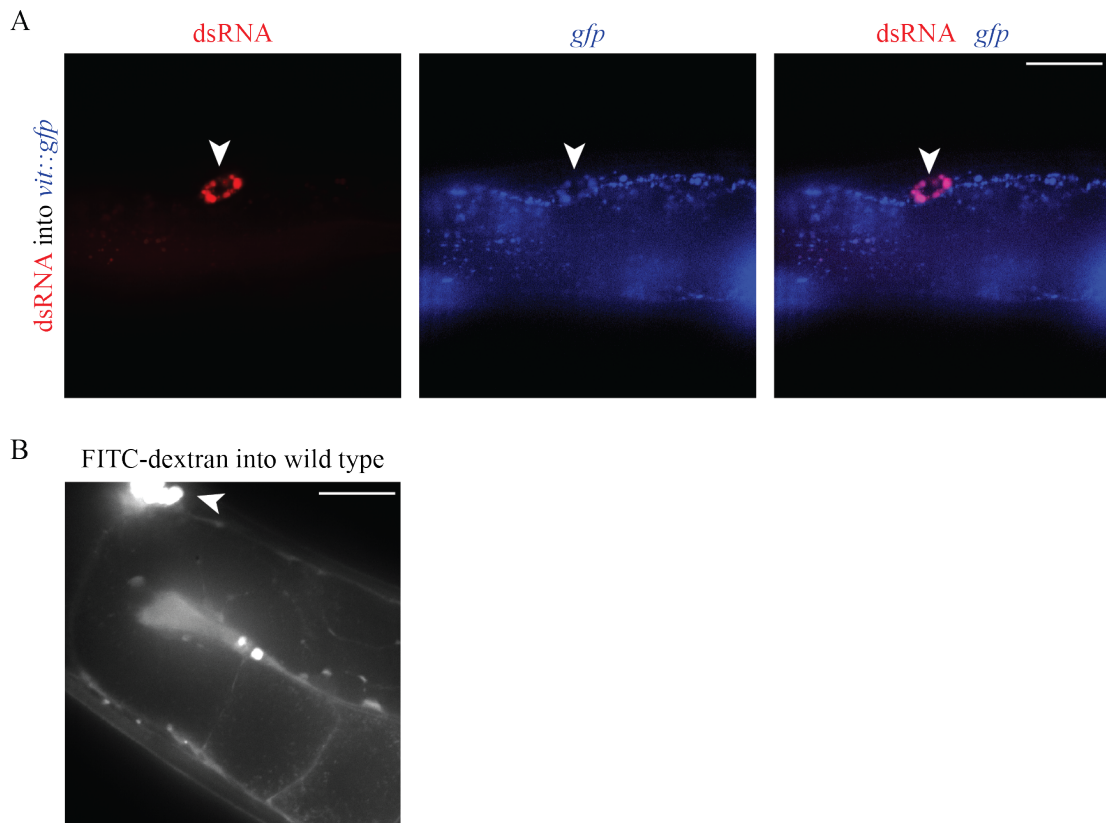


Fig. 3-6. Fluorescent dsRNA, Vit-2::GFP, and dextran accumulate in coelomocytes. (A) Injected fluorescently-labeled dsRNA (*top*) and expressed Vitellogenin-2::GFP (*middle*) accumulate in coelomocytes (*bottom*, merge). (B) Injected FITC-labeled dextran accumulates in coelomocytes. White arrowheads indicate coelomocyte location. Scale bars, 20 μ m. Multiple adults ($n = 4$ (A) and $n = 3$ (B)) were imaged for each experiment.

3.4.5 dsRNA, dextran, and vitellogenin accumulate in large clusters between embryos in the uterus

In *C. elegans*, vitellogenin proteins are synthesized in intestines and then secreted into the pseudocoelomic fluid that fills the body cavity (64). While some vitellogenin proteins are endocytosed into oocytes, others can be observed in the lumen of the uterus in lipid droplets that have been proposed to be proportionate in size to the concentration of extracellular vitellogenin (85). Consistently, we detected vitellogenin droplets in the uterus of uninjected animals expressing *vit-2::gfp* (Fig. 3-7 *A*). Upon injecting into the body cavity, we observed the association of dsRNA and dextran in droplets within the uterus (Fig. 3-7 *B* and *C*) and dsRNA and vitellogenin both accumulated within the same droplets (Fig. 4-6, asterisk), suggesting that many kinds of particles in the extracellular space may accumulate in these uterine clusters. Interestingly, these droplets are about 10 μm in diameter (Fig. 3-7 *B* and *C*) in injected animals but are only about 5 μm in diameter in uninjected animals (Fig. 3-7 *A*), suggesting that the amount of extracellular fluid available impacts droplet size.

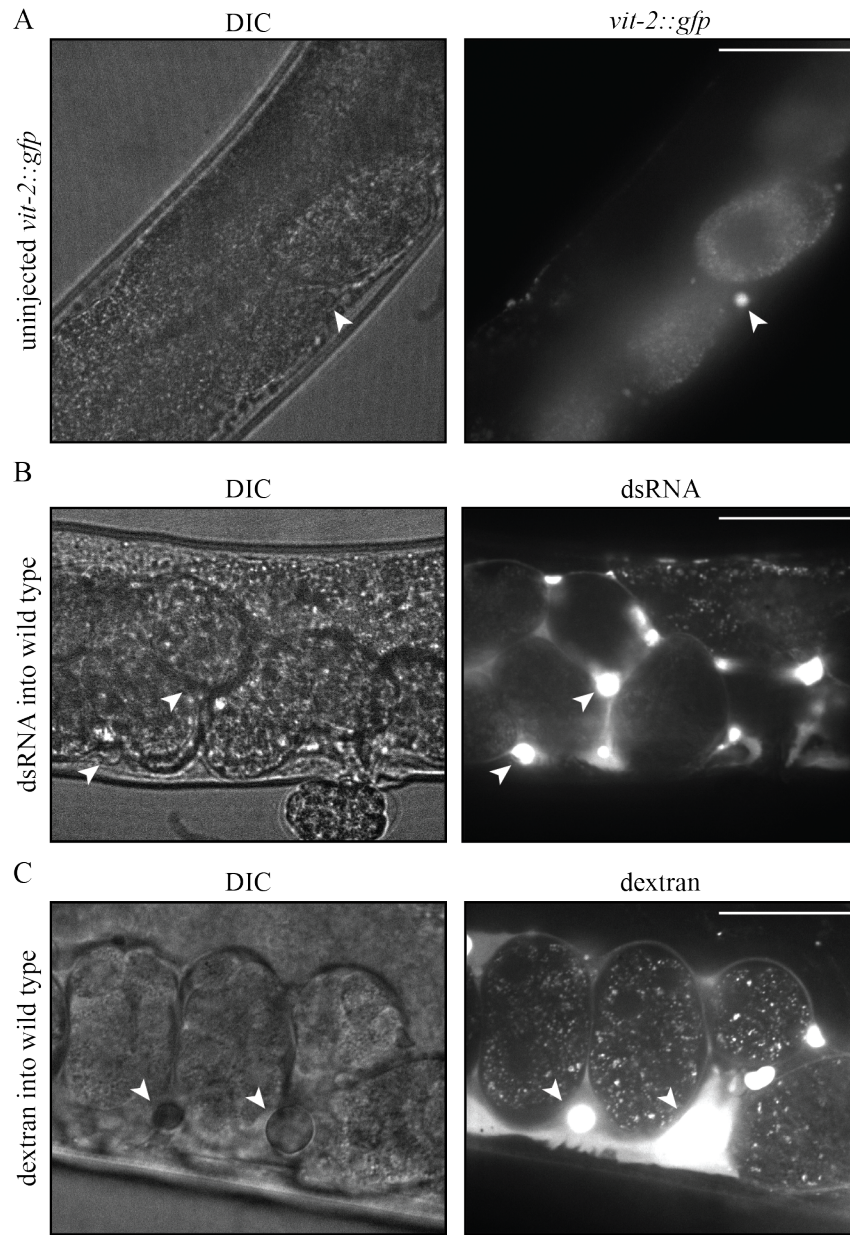


Fig. 3-7. VIT-2, dsRNA, and dextran concentrate in large droplets between embryos in the uterus. (A) VIT-2::GFP collects in a droplet between embryos in the uterus of uninjected animals. (B and C) Fluorescent dsRNA (B) and dextran (C) injected into the body cavity of wild type adult animals collect in droplets in the uterus. White arrowheads indicate the large droplets in the uterus. Scale bars, 50 μm.

3.4.6 Extracellular dsRNA can reach embryos without SID-1-dependent entry

Despite import into oocytes through RME-2-mediated endocytosis, dsRNA is expected to require SID-1 for entry into the cytosol and subsequent silencing. Injection of fluorescent dsRNA into wild-type animals resulted in silencing in embryos (Fig. 3-2), consistent with cytosolic entry either in oocytes or in the embryo. To determine if extracellular dsRNA can reach embryos without cytosolic entry as predicted by genetic analyses (Fig. 2-12 *B*), we injected fluorescently-labeled *gfp*-dsRNA into the body cavity of *sid-1(-)* animals that express GFP in embryos, and imaged the germline of injected animals as well as embryos laid by the injected animals (as schematized in Fig. 3-2 *D*). The dsRNA accumulated in proximal oocytes of *sid-1(-)* adults (Fig. 3-8 *A*), as was observed in wild-type animals. The dsRNA also accumulated in embryos, but unlike in wild-type embryos, such accumulation was not accompanied by silencing of *gfp* expression in *sid-1(-)* embryos (Fig. 3-8 *B-E*). Thus, extracellular dsRNA can be imported into oocytes and can reside in presumed intracellular vesicles that reach embryos without SID-1-dependent entry into the cytosol.

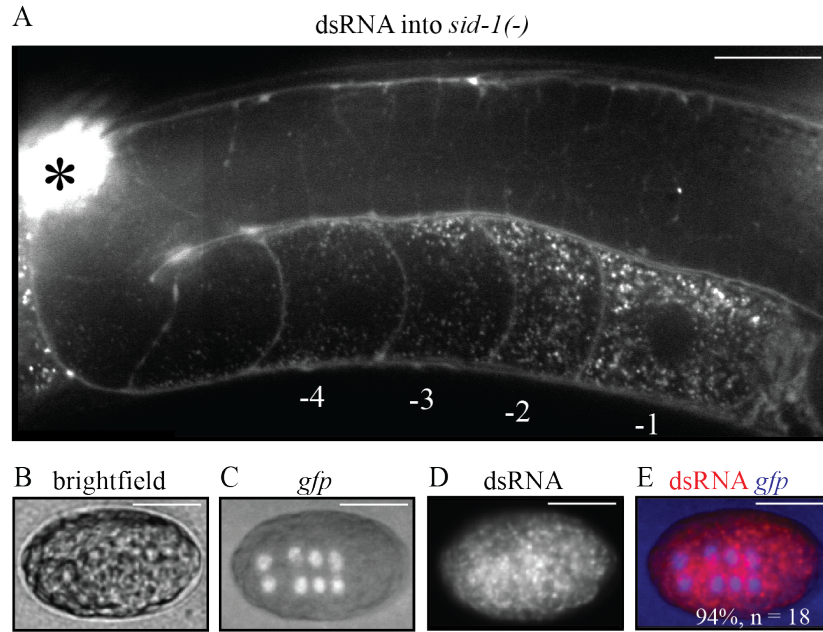


Fig. 3-8. Extracellular dsRNA can accumulate without cytosolic entry in proximal oocytes and subsequently within embryos. (A) In injected *sid-1(-)* animals, dsRNA concentrated in proximal oocytes (-1 and -2). Slices from Z-series (movies 8 to 9) were spliced. Asterisk indicates brightly fluorescent coelomocyte. (B-E) *sid-1(-)* embryos inherited the *gfp*-dsRNA but no silencing of *Pend-1::gfp* occurred. Scale bars, 20 μ m and proximal oocytes are numbered as in Fig. 4. Multiple adults (n = 4 in (A)) and embryos (n = 18 in (B-E)) were imaged for each experiment.

3.5 Discussion

In this chapter, we further developed the model proposed in Chapter 2 with genetic analyses (Fig. 2-14) by using cell biological approaches. We observed the direct inheritance of extracellular dsRNA to progeny by uptake into oocytes from the extracellular space (Fig. 3-9). We injected fluorescently-labeled dsRNA into the extracellular space and detected the accumulation of dsRNA in proximal oocytes (Fig. 3-2 E and Fig. 3-9 A) independent of the dsRNA importer SID-1 (Fig. 3-8 A). The dsRNA that accumulated in oocytes could be inherited to progeny (Fig. 3-2 F-I)

and could silence a gene of matching sequence when SID-1 was present (Fig. 3-2 *F-I* and Fig. 3-8 *B-E*). dsRNA entry into oocytes was dependent upon RME-2-mediated endocytosis (Fig. 3-5 *A*) and coincided with vitellogenin entry into oocytes (Fig. 3-5 *A* and Fig. 3-9 *B*). dsRNA and vitellogenin also appeared to associate together in the extracellular space in droplets within the uterus (Fig. 3-7).

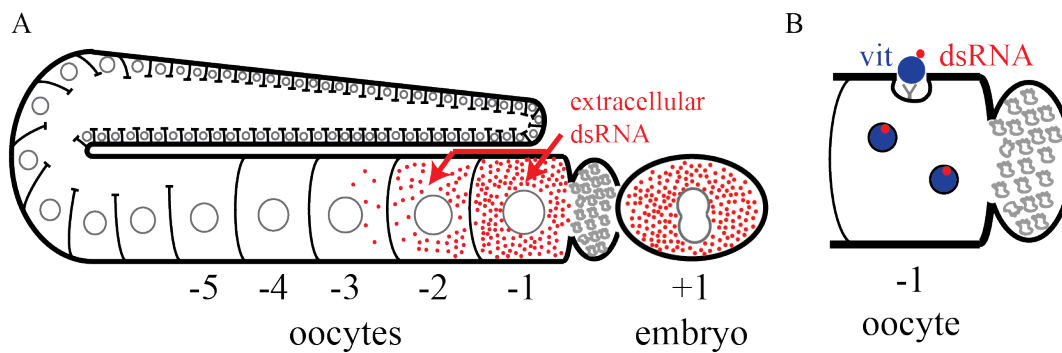


Fig. 3-9. Model: Extracellular dsRNA can be transported along with vitellogenin through oocytes to progeny. (A) Extracellular dsRNA (red) accumulates in the proximal oocytes (-1 and -2) and is inherited to progeny. (B) Extracellular dsRNA (red) is taken up into oocytes along with vitellogenin (blue) by receptor-mediated endocytosis.

3.5.1 Bulk endocytosis of materials from the extracellular space into oocytes

Extracellular dsRNA, vitellogenin, and dextran accumulated in oocytes and progeny embryos (Fig. 3-2 *E-I*, Fig. 3-5, and Fig. 3-7 *C*). Accumulation of dsRNA and vitellogenin in oocytes was dependent upon RME-2-mediated endocytosis (Fig. 3-5 *A*). Together, these results suggest that during RME-2-mediated endocytosis other components present in the extracellular fluid, in addition to vitellogenin, can be taken up by oocytes and reach progeny. This is an intriguing result as this suggests that the

composition of the extracellular fluid can affect progeny. In larger nematode species, the pseudocoelomic fluid is at a neutral pH and contains proteins, fats, and other chemical compounds (86). Vitellogenin fractions from *C. elegans* also contained two RNA species, suggesting that nucleic acids may be present in the extracellular fluid (87). Nucleic acids from the environment may also be present in the pseudocoelom as ingested dsRNA can cross the intestine into the fluid-filled body cavity that surrounds all tissues including the germline without SID-1-dependent entry into the cytosol of intestinal cells (51, 57, 79). Determining the constituents of the extracellular fluid will be an essential next step in understanding what can reach progeny from parents.

3.5.2 dsRNA entry into the germline

Surprisingly, dsRNA was not detected within the rachis of the germline (Fig. 3-2 *E* and Fig. 3-8 *A*). However, it is possible to silence genes in the germline by introducing dsRNA outside the germline (Fig. 1-3). It is possible that 50 bp dsRNA is unable to enter the germline. Consistently, 100 bp but not 50 bp *mex-3*-dsRNA injected outside the germline caused silencing of *mex-3* in the germline (39).

3.5.3 dsRNA entry into somatic tissues

Fluorescently-labeled dsRNA could also be detected in somatic cells, however, entry into somatic cells is likely to be different from entry into oocytes as shown here, because RME-2 can only be detected in the oocytes of the germline and not in somatic cells (66).

3.5.4 Vitellogenin and dsRNA in the extracellular space

Vitellogenin and dsRNA accumulated in droplets between embryos within the uterus (Fig. 3-7 *A* and *B*). Dextran also accumulated in droplets within the uterus (Fig.

3-7 C), suggesting that the interactions between vitellogenin and dsRNA may not be specific. It has been proposed that these droplets expand or contract depending on the amount of vitellogenin in the extracellular fluid (85). Consistent with this, increasing the volume of the extracellular fluid by injecting dsRNA or dextran in buffer into the extracellular fluid appears to increase the size of the droplets (Fig. 3-7). Molecules in the body cavity may collect in droplets within the uterus due to the suspected microenvironments produced by the extracellular matrix in the uterus.

3.5.5 Implications for the location of SID-1 in cells

The inheritance of extracellular dsRNA by RME-2-mediated uptake in oocytes independent of SID-1 in parents (Fig. 3-9) suggests that dsRNA is transported to progeny within a vesicle. For inherited dsRNA to enter the cytosol of cells and initiate silencing, dsRNA must be imported into the cytosol of cells through SID-1. Therefore, SID-1 is likely to be present on an intracellular vesicle to enable dsRNA import into cells. However, it is also possible that the intracellular vesicles containing inherited dsRNA fuse with the cell membrane, releasing dsRNA outside the cell and then dsRNA is imported into the cell through SID-1 present on the cell membrane.

Chapter 4: Inherited RNA in the embryo

4.1 Preface

Fig. 4-6 was published with some modifications in: Marré JA, Traver EC, and Jose AM (2016) Extracellular dsRNA is transported from one generation to the next in *C. elegans*. *Proceedings of the National Academy of Sciences USA* 113(44):12496-501.

All data in this chapter was generated by Julia Marré.

Some worm strains were obtained from the *Caenorhabditis elegans* Genetic stock Center and the Seydoux laboratory (Johns Hopkins University). The Hamza laboratory (University of Maryland) provided some of the bacteria that express dsRNA. The Andrews laboratory (University of Maryland) trained me and allowed me to use their confocal microscope.

4.2 Introduction

In Chapters 2 and 3, we used genetic and cell biological approaches to provide evidence that extracellular dsRNA can be transported through oocytes to silence genes in progeny. Here, we investigate inherited dsRNA in the embryo by following the fate of fluorescently-labeled dsRNA in the embryo. We also compare the path of inherited dsRNA with inherited vitellogenin during embryogenesis.

We found that inherited dsRNA separates from inherited vitellogenin during early embryogenesis. During late embryogenesis, inherited dsRNA accumulates in the gut where inherited vitellogenin also accumulates.

4.3 Materials and methods

4.3.1 Strains, transgenesis, and oligonucleotides

All strains used are listed in Table 4-2 and all oligonucleotides used are listed in Table 4-1. Strains were cultured and maintained as described in (70).

Table 4-1. Strains used.

Strain	Genotype
N2	wild type
RT130	<i>pwIs23</i>
TX691	<i>unc-119(ed3) III; telIs46</i>

Table 4-2. Oligonucleotides used (5' to 3', IDT).

Oligonucleotide	Sequence
P1 (<i>gfp</i> sense RNA)	ugguccuucuugaguuuuguaacagcugcugggauacaca uggcauggau
P2 (5' Atto 565 labeled <i>gfp</i> antisense ssRNA)	5' Atto 565-auccaugccauguguaaucccagcagcuguu acaaacucaagaaggacca
P3 (5' Atto 488 labeled <i>gfp</i> sense ssRNA)	5' Atto 488-ugguccuucuugaguuuuguaacagcugcug ggauuacacauggcauggau

4.3.2 Injection of dsRNA and dextran

Annealing fluorescently-labeled *gfp*-dsRNA: ssRNA oligos P1 and P2 or P2 and P3 were resuspended in nuclease free 1X TE (IDT) to ~1635 ng/μl. Equal

volumes of each ssRNA were mixed in nuclease free duplex buffer (IDT) to a final concentration of ~490 ng/μl of each ssRNA. This mixture was heated to 95°C and cooled at 1 min/degree to 10°C, resulting in ~980 ng/μl of dsRNA. P1, P2, and the annealed dsRNA (Fig. 3-1) or P2, P3, and the annealed dsRNA (Fig. 4-1) were run in a 12% denaturing polyacrylamide gel. Gels were first imaged with the Typhoon Trio Variable Mode Imager (GE Healthcare) using a 532 nm laser and then stained with ethidium bromide (amresco) followed by imaging with the Molecular Imager Gel Doc XR (Bio-Rad) using ultraviolet light.

Injection: Adult animals (24 hours post L4-stage) were injected with ~325-980 ng/μl *gfp*-dsRNA into the body cavity past the bend of the posterior arm of the gonad. Atto 488/Atto 565-labeled *gfp*-dsRNA was injected into the body cavity for experiments shown in Fig. 4-2, Fig. 4-4, Fig. 4-7, and Fig. 4-9. Atto 565-labeled *gfp*-dsRNA was injected into the body cavity for experiments shown in Fig. 4-6.

4.3.3 Fluorescence imaging

Embryos from adults injected with fluorescent *gfp*-dsRNA: Embryos were imaged either 3 hours post injection (Fig. 4-2, Fig. 4-4, and Fig. 4-6) or 22 hours post injection (Fig. 4-7 and Fig. 4-8). Laid embryos or adults containing embryos were picked off plates and placed into 10 μl or 5 μl of 3 mM levamisole on a coverslip, respectively, and let sit for at least 5 minutes before placing on a 2% agarose pad on a slide. Embryos were imaged using the Eclipse Ti Spinning Disk Confocal (Nikon) with the 60X objective lens. Atto 565 was excited using a 561 nm laser and fluorescence was collected through a 415-475 nm and 580-650 nm emission filters. Atto 488/GFP was excited using a 488 nm laser and fluorescence was collected

through a 415-475 nm and 580-650 nm emission filter. Images were adjusted for display using ImageJ (NIH).

4.3.4 pH assay

1 μ l of Atto 488/Atto 565-labeled *gfp*-dsRNA at 325 ng/ μ l was added to 19 μ l of nuclease free duplex buffer (IDT) at different pH on parafilm. Droplets were imaged using an AZ100 microscope (Nikon) with a Cool SNAP HQ² camera (Photometrics). A C-HGFI Intensilight Hg Illuminator was used to excite GFP (filter cube: 450-490 nm excitation, 495 dichroic, and 500-550 nm emission) or DsRed/Atto 565 (filter cube: 530-560 nm excitation, 570 dichroic, and 590-650 nm emission). Exposure times were scaled for to just under saturation for pH 12 and then all droplets were imaged using the same exposure time. Images were adjusted for display using ImageJ (NIH).

4.4 Results

4.4.1 Doubly-labeled extracellular dsRNA can be inherited and can silence a gene of matching sequence in progeny

gfp-dsRNA labeled with one fluorophore on the 5' end of the antisense strand injected into the body cavity of worms can be inherited and can silence a *gfp* gene in progeny (Chapter 3). However, labeling only one end of the dsRNA molecule may prevent the elucidation of strand specific mechanisms, so we injected 50-bp *gfp*-dsRNA labeled with two different fluorophores at each 5' end (Fig. 4-1, Atto 488 and Atto 565). Upon injection into the body cavity of transgenic worms with embryonic expression of *gfp*, doubly-labeled *gfp*-dsRNA was detected in progeny embryos (Fig.

4-2). Embryos containing inherited doubly-labeled dsRNA were also silenced for *gfp* in embryos as early as the ~16-cell staged embryo (Fig. 4-2). While both fluorophores labeling the dsRNA could be detected in progeny, there is significant background fluorescence in uninjected embryos using the imaging conditions for Atto 488 (Fig. 4-3), thus making it difficult in some embryos to determine the difference between background fluorescence and presence of Atto 488.

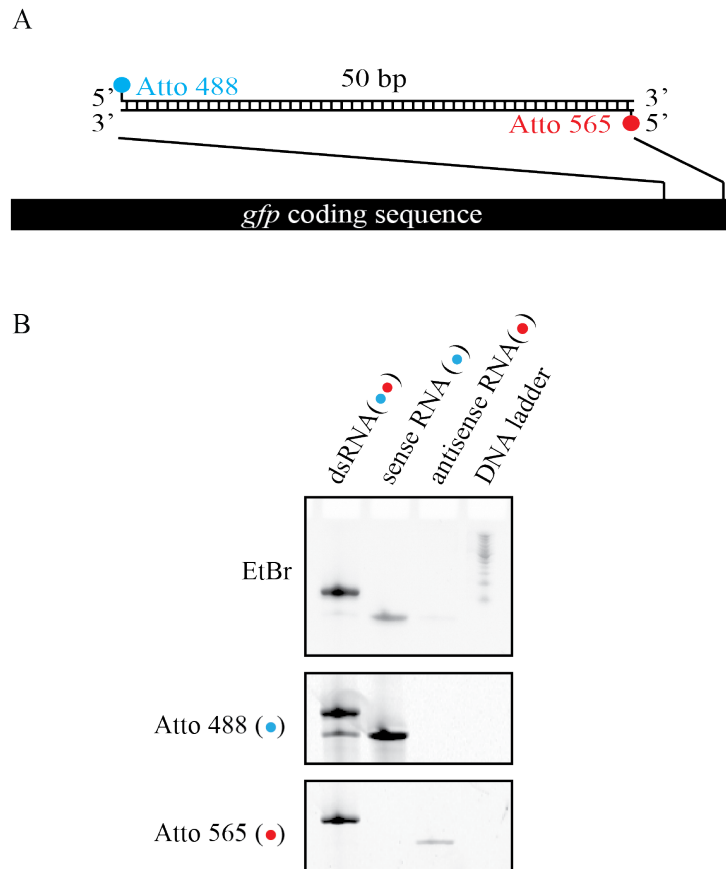


Fig. 4-1. Annealing Atto 488-labeled sense RNA and Atto 565-labeled antisense RNA generates fluorescent dsRNA. (A) Schematic of fluorescent *gfp*-dsRNA. The Atto 488 label is attached to the 5' end of the sense strand of dsRNA and the Atto 565 label is attached to the 5' end of the antisense strand of dsRNA. (B) RNA was run in a 12% polyacrylamide gel and imaged for fluorescence (*middle*, Atto 488 and *bottom*, Atto 565) and stained with ethidium bromide (*top*, EtBr). Low molecular weight DNA ladder was run in the fourth lane ranging from 25 bp (bottom band) to 766 bp (top band). Note: the concentration of RNA loaded into each lane varies (lower concentration in antisense RNA lane than sense RNA lane).

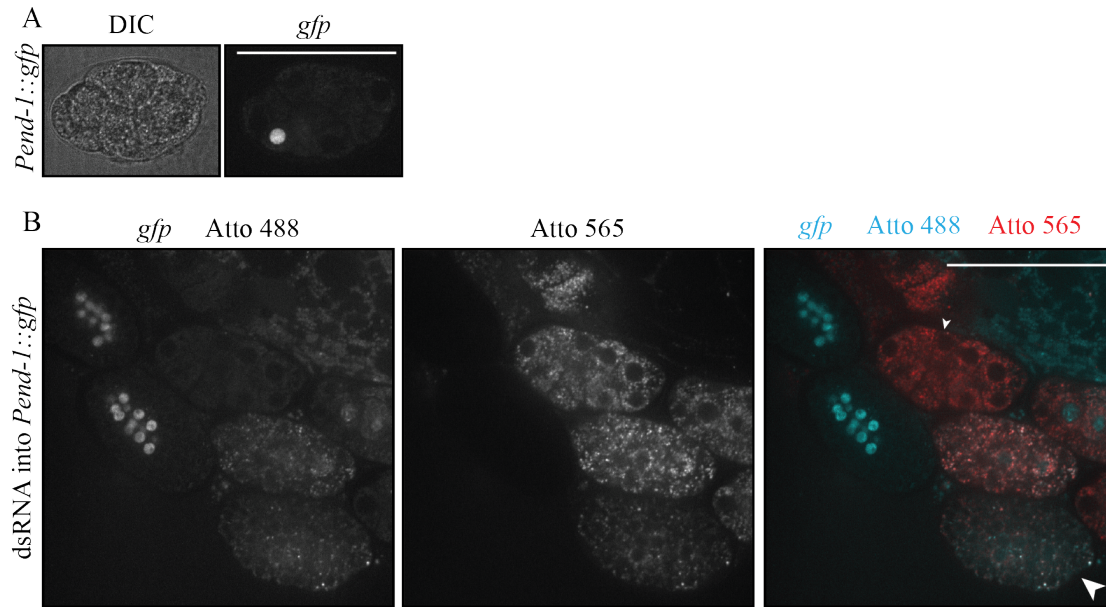


Fig. 4-2. Extracellular doubly-labeled dsRNA can be inherited and can cause silencing in progeny. (A) Uninjected embryo expressing *Pend-1::gfp*. One slice of Z-stack shown here so only one gut cells can be viewed but there are two gut cells expressing *gfp* in this embryo. (B) Embryos with inherited doubly labeled dsRNA show silencing of *gfp*. Embryos are expressing *Pend-1::gfp* from parents injected with doubly-labeled dsRNA (Atto 488 and Atto565) in the body cavity. Large arrowhead points to an embryo with inherited dsRNA that is older/more developed than the embryo being pointed to with the small arrowhead. Scale bars, 50 μ m; n = 3 in (A) and n > 15 in (B).

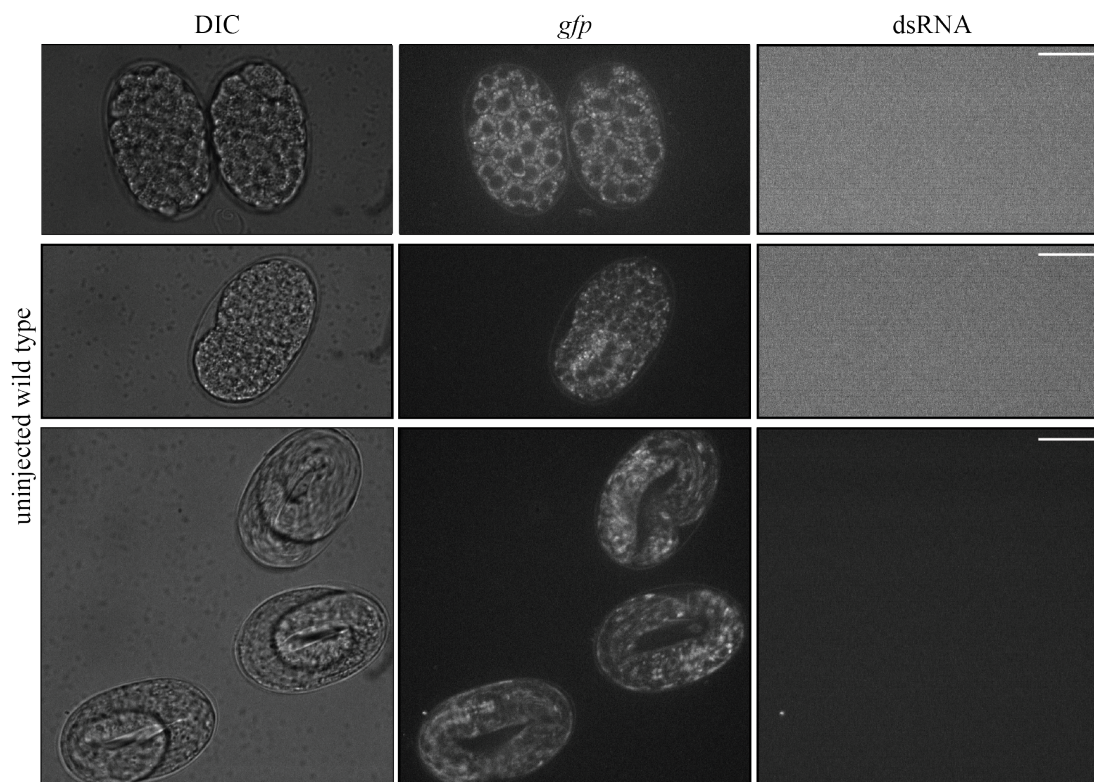


Fig. 4-3. Uninjected embryos have high background autofluorescence when imaged using optimal conditions for Atto 488. Wild-type embryos imaged using optimal conditions for Atto 488 and Atto 565 display high background autofluorescence in conditions for Atto 488 (*middle*) but not Atto 565 (*right*). Scale bars, 20 μm ; $n = 7$.

4.4.2 Inherited doubly-labeled dsRNA is a different color in embryos depending on the time the embryo was fertilized

Extracellular doubly-labeled *gfp*-dsRNA can be detected in progeny (Fig. 4-2). However, when we imaged embryos about 3 hours post injection and looked at the merged image for both fluorophores, we noticed that the more developed embryos (Fig. 4-2, large arrowhead, and Fig. 4-4 *A*, *bottom*) were a different color than the less developed embryos (Fig. 4-2, small arrowhead, and Fig. 4-4 *A*, *top*). By measuring the brightness value for each fluorophore in each Z-slice and plotting the fluorescence

ratio, we found that more developed embryos differed from the less developed embryos by 5-fold (Fig. 4-4 *B*). This difference in fluorescence ratio between embryos of differing ages could be due to the pH maturation of intracellular compartments containing inherited dsRNA (as proposed to be the method of transport of dsRNA to progeny in Chapters 2 and 3), a bias in the amount and/or type of dsRNA different oocytes receive (e.g., the -1 oocyte ovulating upon injection of dsRNA into the body cavity receives different dsRNA than the -1 oocyte ovulating 1 hour post injection), or a difference in the level of background autofluorescence between embryos of differing age. To assess the effect pH maturation could have on the fluorophores labeling the dsRNA, we placed the doubly-labeled dsRNA in different pH solutions, imaged each fluorophore, and plotted the fluorescence ratio normalized to pH 7 (Fig. 4-5). We did not observe any 5-fold changes in the fluorescence ratio for the pH solutions tested (Fig. 4-5), suggesting that pH maturation alone cannot explain the differences observed in embryos. However, there appears to be a discrepancy between the color of the 488/565 merged pH droplets and the fluorescence ratio measured (e.g., the measured fluorescence ratio for pH 4 and pH 6 is comparable in Fig. 4-5 *B* but the colors of the merged images in Fig. 4-5 *A* are different). Further investigation is needed to explain this inconsistency.

To evaluate whether there is a bias in the amounts/types of dsRNA different oocytes receive, single embryos will need to be imaged over time as soon as fertilization occurs and compared with embryos fertilized at varying times post injection of dsRNA into the body cavity.

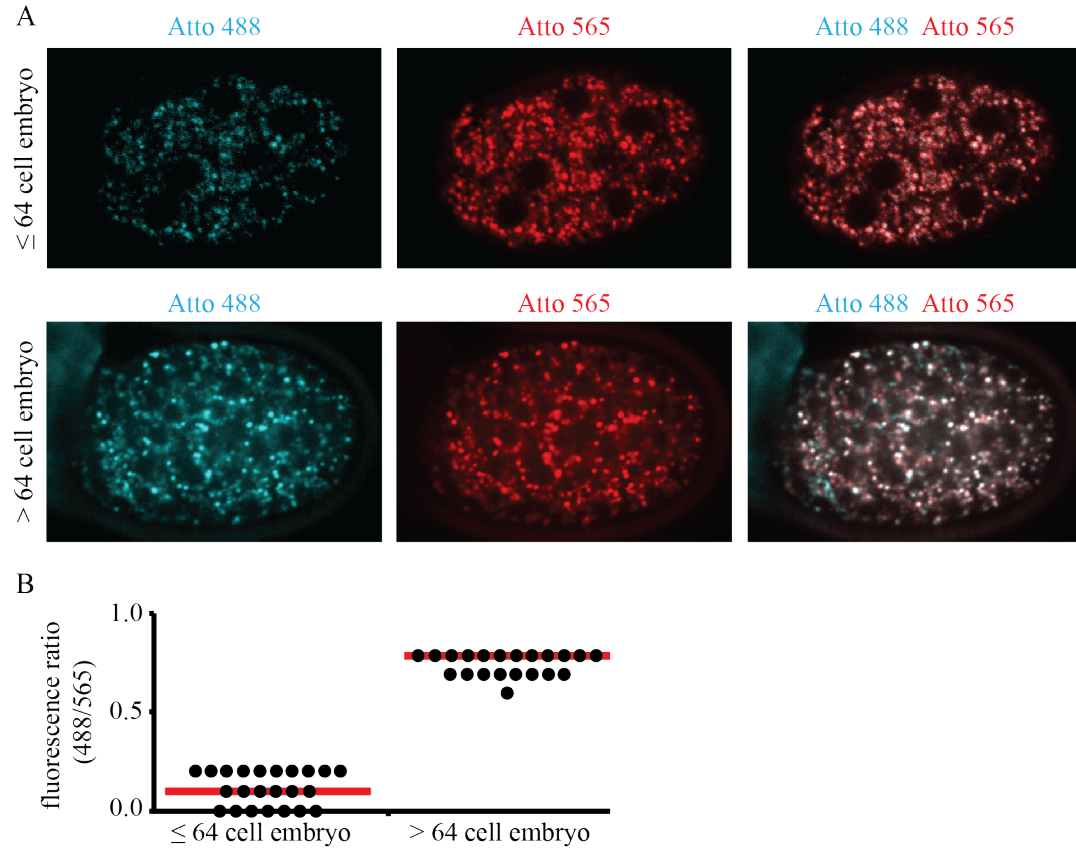


Fig. 4-4. Inherited doubly-labeled dsRNA is a different color in embryos depending on the time the embryo was fertilized. (A) Inherited doubly-labeled dsRNA is a different merged color in embryos less than 64 cells (*top*) compared with embryos greater than 64 cells (*bottom*). Representative images depicted. (B) Fluorescence ratio for each Z-slice of embryos in A. $n = 28$ ≤ 64 -cell embryo and $n = 6$ > 64 -cell embryo in (A) and $n = 1$ embryo for each category in (B).

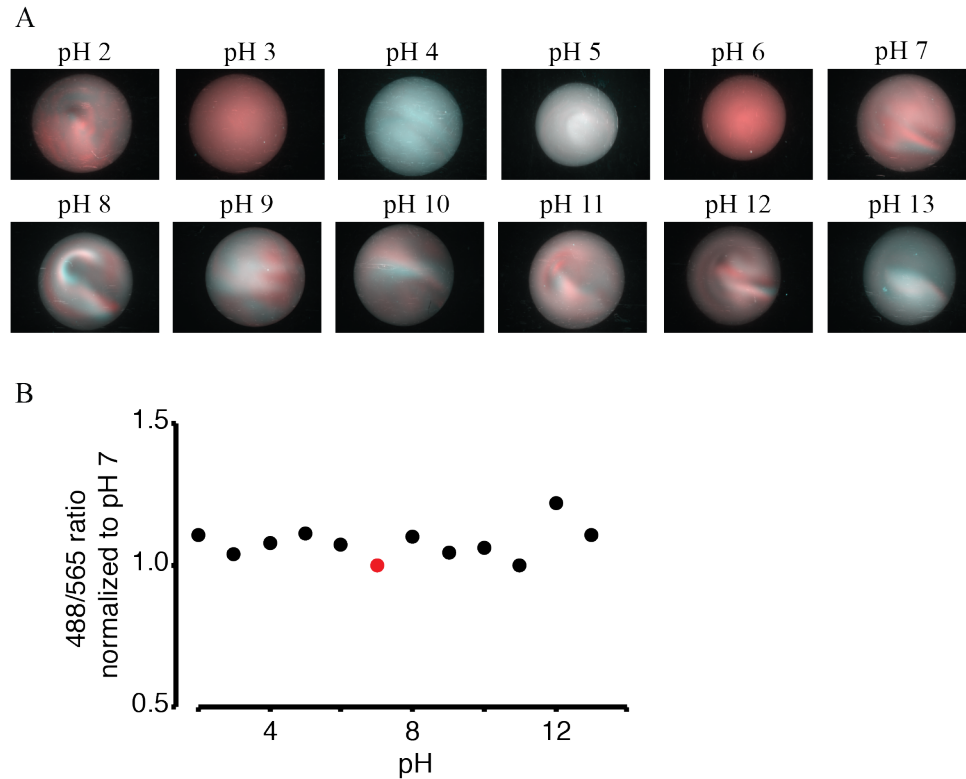


Fig. 4-5. pH does not change the fluorescence ratio of Atto 488 to Atto 565 by 5-fold. (A) Doubly-labeled dsRNA in different pH buffers. Atto 488 and Atto 565 channels merged for each pH. (B) Fluorescence ratio normalized to pH 7 for each image in A.

4.4.3 Inherited dsRNA and vitellogenin appear to separate during early embryo development

Extracellular dsRNA and vitellogenin are both endocytosed into oocytes by RME-2 dependent receptor-mediated endocytosis where dsRNA and vitellogenin accumulate in intracellular puncta (Chapter 3, Fig. 3-5). However, during early embryo development inherited dsRNA and vitellogenin appear to separate (Fig. 4-6), suggesting that vitellogenin and dsRNA are trafficked differently in early embryos. These results raise an intriguing hypothesis that is worthy of further investigation: C.

C. elegans embryos have mechanisms to distinguish inherited nutrition (vitellogenin) from inherited information (dsRNA) when both reside within the same putative intracellular vesicle. However, because we performed live imaging of embryos, it is possible that the apparent separation of inherited dsRNA and vitellogenin can be explained by the movement of particles live imaging, resulting in what looks like separation. Comparing the location of inherited dsRNA and vitellogenin in live imaging embryos with fixed embryos will further resolve these possibilities.

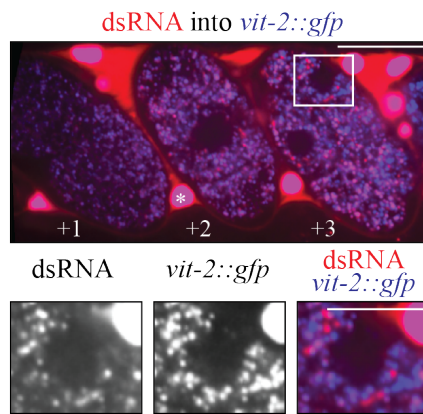


Fig. 4-6. dsRNA and vitellogenin separate during early embryo development. Colocalization of fluorescently labeled dsRNA and VIT-2::GFP is reduced as the embryo develops. Single confocal slice of an adult animal showing accumulation of fluorescently labeled dsRNA that was injected into the body cavity of a strain with *vit-2::gfp* in embryos held *in utero* ($n = 6$ <4-cell embryos and $n = 10$ \geq 4-cell embryos). *Top*, Merged image showing +1, +2, and +3 embryos after fertilization (Scale bar, 20 μ m); *Bottom*, zoomed image of highlighted region (white box) in top image for individual channels of dsRNA and VIT-2::GFP fluorescence and merge (Scale bar, 10 μ m). Asterisk indicates droplets in the uterus containing dsRNA and VIT-2::GFP (also seen in Fig. 3-7).

4.4.4 Inherited dsRNA and vitellogenin accumulate in gut cells during development

In *C. elegans* embryos, vitellogenin is secreted by blastomeres and re-imported by intestinal cells, resulting in enrichment within the intestinal primordium (specifically, gut granules and gut lumen (88-90). To determine the fate of inherited dsRNA later in development, we injected doubly-labeled dsRNA into the body cavity of parent worms and imaged progeny of various stages 22 hours later (Fig. 4-7 and Fig. 4-8). While we observed a separation of inherited dsRNA and vitellogenin in embryos during early development (Fig. 4-6), as development progressed we observed the concentration of inherited dsRNA in gut cells and the gut lumen (Fig. 4-7 and Fig. 4-8), similar to what has been observed for vitellogenin (88-90). As previously noted, it is difficult to distinguish background autofluorescence from Atto 488 label on dsRNA so dsRNA can only confidently be followed via the Atto 565 fluorophore. However, the background autofluorescence observed highlights gut granules, and therefore gut cells later during development, and can be used as an anatomical marker to determine the location of inherited dsRNA (Atto 488 channel for location of gut cells and Atto 565 channel for location of inherited dsRNA in Fig. 4-7 and Fig. 4-8). We also noticed that younger progeny (embryos laid the latest) did not contain inherited dsRNA (Fig. 4-7, top two embryos), consistent with previous experiments where silencing in progeny was limited to the earliest laid progeny (Fig. 2-1 C). These results suggest that the bulk of inherited dsRNA follows the same fate as inherited vitellogenin in the embryo.

Although silencing was observed in the 16-cell staged embryo, the majority of inherited dsRNA still appears to be within intracellular puncta (Fig. 4-2) and inherited dsRNA can still be detected as late as L1 animals (Fig. 4-9 *B* and data not shown). This suggests that either inherited dsRNA can persist and avoid entering cells to be processed or the fluorescence detected is not dsRNA and is label alone. Further work is required to distinguish between these two possibilities by performing experiments such as *in situ hybridization* or immunofluorescence to detect RNA in embryos.

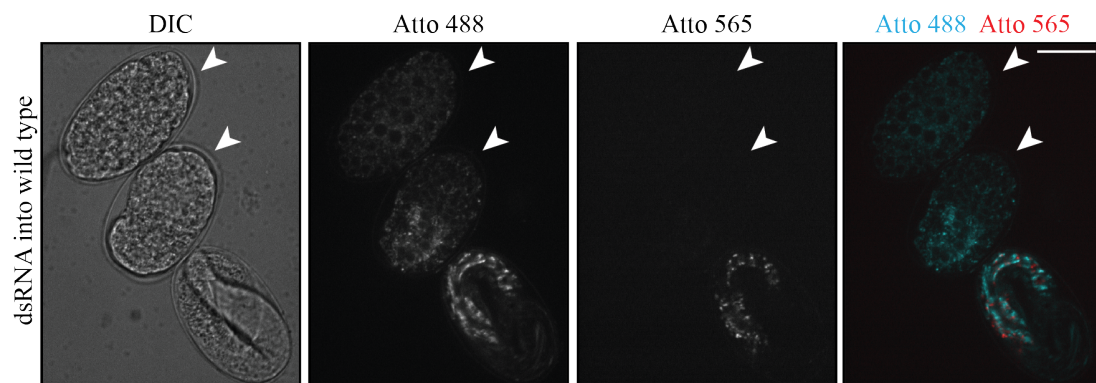


Fig. 4-7. Inherited dsRNA accumulates in gut cells during late embryogenesis. Representative images of inherited doubly labeled dsRNA (Atto 488 and Atto 565) accumulating in gut cells in wild-type embryos laid 22 hours after parents were injected with dsRNA. Arrowheads indicate embryos without inherited dsRNA. Scale bar, 20 μ m. $n = 7$ embryos with inherited dsRNA and $n = 9$ embryos without inherited dsRNA.

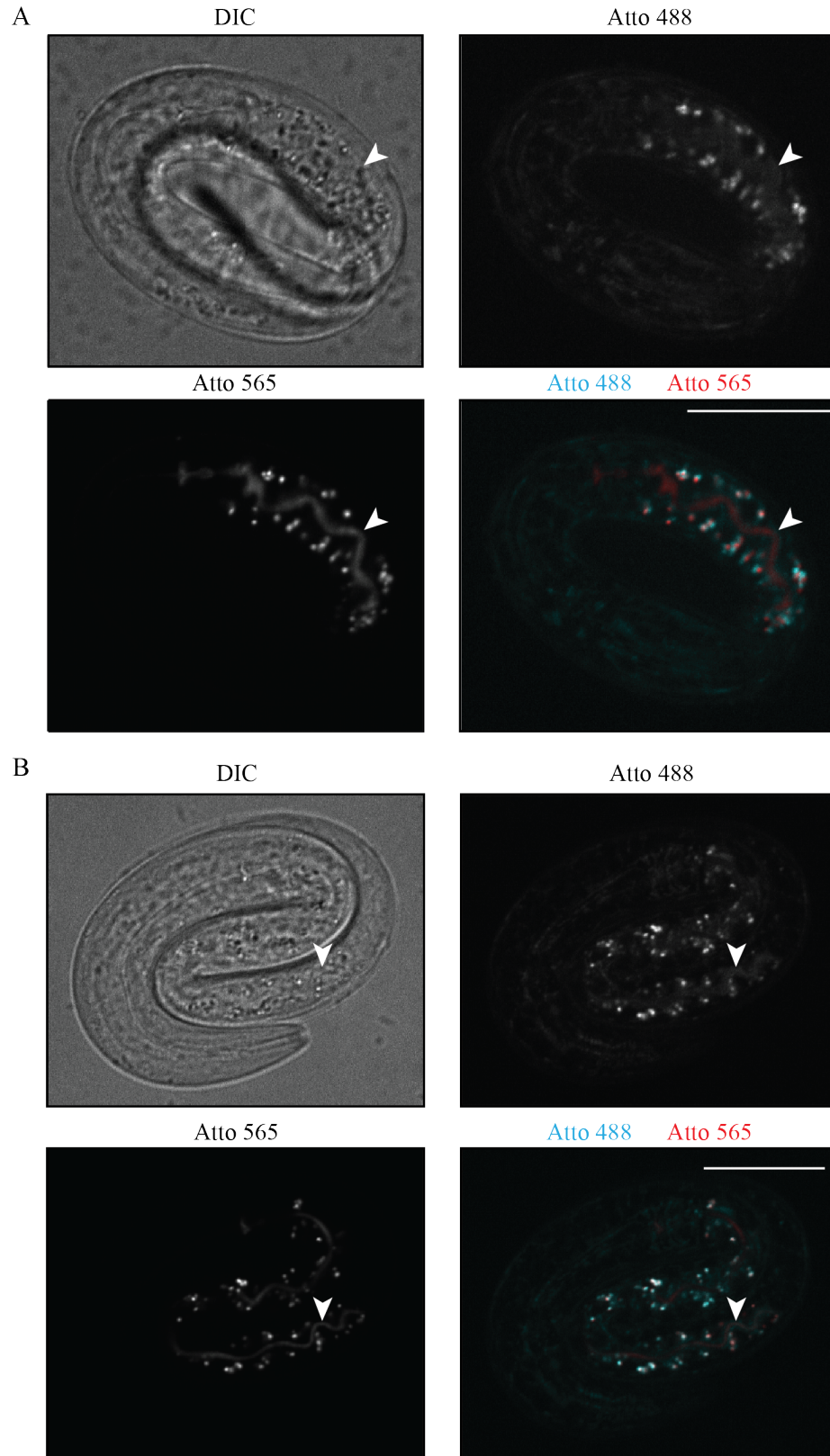


Fig. 4-8. Inherited dsRNA accumulates in the gut cells and the gut lumen in late-staged embryos. Representative images of inherited doubly labeled dsRNA (Atto

488 and Atto 565) accumulating in gut cells and the gut lumen of wild type embryos laid 22 hours after parents were injected with dsRNA Arrowheads indicate gut lumen. Scale bars, 20 μ m. n = 7 embryos with inherited dsRNA.

4.5 Discussion

In this chapter, we have elucidated some of the dynamics of inherited RNA in the embryo. Fluorescently-labeled *gfp*-dsRNA in the extracellular space with both Atto 488 and Atto 565 at either 5' end of the dsRNA molecule (Fig. 4-1) accumulated in progeny and was capable of silencing a *gfp* gene in the 16-cell staged embryo (Fig. 4-2). Inherited dsRNA and vitellogenin were observed to begin development in the same intracellular puncta (Fig. 4-6, +1 and +2 embryo, and Fig. 2-9 A) but then the two components separated as early embryogenesis occurred (Fig. 4-6, +3 embryo, and Fig. 2-9 A). However, as development continued to progress, inherited dsRNA was observed in gut cells (Fig. 4-7, Fig. 4-8, and Fig. 2-9 B) and in the gut lumen (Fig. 4-8 and Fig. 4-9 B), where inherited vitellogenin has been seen to accumulate (91),

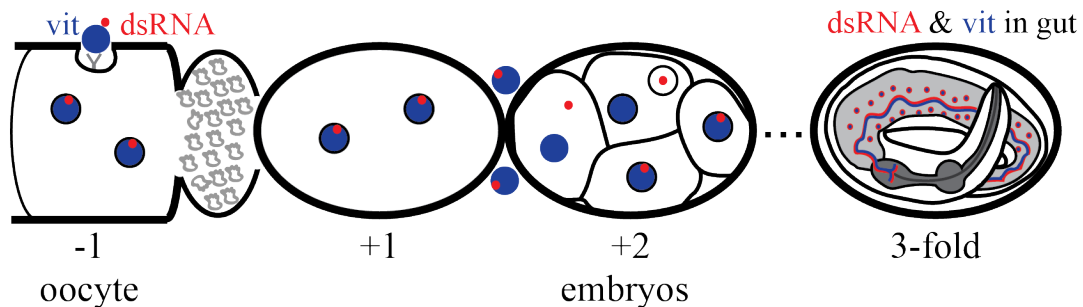


Fig. 4-9. Model: Inherited dsRNA separates from vitellogenin early in development but ultimately accumulates in gut cells where vitellogenin is located later in development. (A) dsRNA (red) and vitellogenin (blue) are taken up by oocytes and remain in the same intracellular vesicles during early embryogenesis (+1 embryo). As development progresses, some inherited dsRNA (red) and vitellogenin (blue) separate (+2 embryo). This separation may occur in the cytosol or in different intracellular vesicles. (B) By the 3-fold stage of embryogenesis, inherited dsRNA (red) and inherited vitellogenin (blue) accumulate in gut granules (dots) and within the gut lumen (lines).

4.5.1 Implications for dsRNA transport through SID-1

C. elegans SID-1 has been proposed to function as a multimer (92) and structural analyses of the extracellular domain of human SID-1 suggest that SID-1 assembles as a tetrameric channel capable of transporting dsRNA into cells by end-on transit (93). The ability of inherited doubly-labeled dsRNA to initiate silencing in progeny suggests that doubly-labeled dsRNA is capable of entering the cytosol through SID-1 or one/both fluorophores are cleaved off dsRNA before being transported through SID-1.

4.5.2 Insights into when dsRNA can enter the cytosol

Inherited dsRNA silenced a gene that begins expressing in the 16-cell staged embryo (Fig. 4-2), suggesting that dsRNA has already entered the cytosol and been processed by this stage. RNA synthesis can first be detected in embryos beginning at the 4-cell stage (94), suggesting that factors necessary for dsRNA transport into cells (e.g., SID-1) and processing within cells (e.g., RDE-4, RDE-1) may be made in embryos to enable silencing by the 16-cell stage when we observed silencing. However, *sid-1*, *rde-4*, and *rde-1* mRNA are all highly abundant in the germline (95-98), so it is possible that these factors are deposited to the embryo from the maternal germline. Silencing by inherited dsRNA will need to be assessed for an earlier expressing gene to further clarify where factors can be expressed for silencing in the embryo.

While inherited dsRNA was capable of silencing a gene in the 16-cell staged embryo (Fig. 4-2), silencing by inherited dsRNA was only assessed for a gene in the gut. It is possible that inherited dsRNA is only capable of entering the gut and to

silence genes in other tissues, dsRNA must spread from the gut to other tissues. Previous work suggests that dsRNA expressed from within early gut cells is capable of spreading between cells through SID-1 in the 60-cell staged embryo (80). It is possible that inherited dsRNA enters the cytosol of the gut and then spreads between cells in a similar way.

The specific cells that inherited dsRNA is capable of entering in the embryo and the time in development this occurs can be very informative for understanding what subsequent cells are exposed to cytosolic inherited dsRNA. For example, if inherited dsRNA could enter the cytosol of the AB cell in the 2-cell embryo, descendants of the AB cell could receive cytosolic inherited dsRNA.

4.5.3 Specific mechanisms to separate inherited dsRNA and vitellogenin

Inherited dsRNA and vitellogenin appear to separate from within the same intracellular puncta in early embryogenesis (Fig. 4-6 and Fig. 4-9 *A*). This suggests that there may be specific mechanisms to separate dsRNA and vitellogenin or that all apparent separation is due to live imaging conditions. If separation does indeed occur, it is possible that dsRNA separates from vitellogenin when vitellogenin is cleaved by proteases in the developing embryo (67, 99). Interestingly, cathepsin L protease (CPL-1) is thought to process vitellogenin at around the 8-12-cell staged embryo (67), similar to when dsRNA and vitellogenin separation can first be detected in embryos.

4.5.4 General mechanisms of uptake in the developing gut

Inherited dsRNA accumulated in gut cells during late embryogenesis (Fig. 4-7, Fig. 4-8, and Fig. 4-9 *B*). This is similar to what has been observed for inherited vitellogenin (91). Interestingly, fluorescent dyes injected into the perivitelline space

in the embryo accumulated in the gut during embryonic morphogenesis (88), resulting in the hypothesis that during development there is a general mechanism to uptake anything present in the perivitelline space into the gut. Inherited vitellogenin, like what we observed with inherited dsRNA, is absent from all non-gut cells in the embryo and increases in intensity in the gut (88), suggesting that vitellogenin from non-gut cells is secreted and then taken up by the gut rather than vitellogenin is eliminated in non-gut cells. Inherited dsRNA may do the same, but further studies that follow single embryos across the entirety of embryogenesis are required to test this hypothesis.

Like inherited vitellogenin, inherited dsRNA accumulated in the gut lumen in late-staged embryos (~3-fold embryo, Fig. 4-8 and Fig. 4-9 *B*) and persisted until L1 larvae (data not shown). One potential hypothesis for how inherited dsRNA accumulates in the gut lumen is that the developing embryo ingests dsRNA from the perivitelline space. As previously postulated, inherited dsRNA in non-gut cells may be secreted into the perivitelline space and taken up into gut cells as is thought to occur with inherited vitellogenin. Inherited dsRNA in the perivitelline space may persist long enough for the developing embryo to then ingest it when the pharynx begins pumping right before the embryo hatches, resulting in inherited dsRNA being present in the gut lumen at the 3-fold stage or the stage before hatching.

Chapter 5: General discussion

5.1 Introduction

Using genetic analyses and fluorescently-labeled RNA, we establish that extracellular dsRNA is imported into oocytes along with vitellogenin and can reach embryos with or without entry into the cytosol. Cytosolic entry of dsRNA from parental circulation in embryos and subsequent spread between cells of dsRNA processed within the parental germline or during early development in progeny results in robust gene silencing. Extracellular doubly-labeled dsRNA accumulated in progeny where silencing occurred in early embryos. Inherited dsRNA and vitellogenin initially appeared to separate in early embryogenesis, but both accumulated in gut cells by late embryogenesis. In addition to accumulation in gut cells in late embryogenesis, inherited dsRNA also accumulated in the gut lumen.

5.2 Implications for the inheritance of RNA silencing

The direct transfer of extracellular and intracellular dsRNA from parents to progeny when parents ingest dsRNA, when dsRNA is injected into parents, or when dsRNA is expressed within neurons in parents demonstrates that the trigger for RNAi is transported between generations in *C. elegans* (Fig. 3-9 A). Therefore, when multigenerational silencing is observed for genes expressed within the germline (Fig. 1-3), the mechanisms that are required for transgenerational stability of silencing could be initiated in progeny – potentially during germline development. Thus, the production of secondary small RNAs and deposition of chromatin modifications proposed to be required for transgenerational gene silencing (33, 43, 75, 100, 101,

102) could be initiated in progeny when parents encounter dsRNA. These considerations also impact the interpretation of experiments evaluating the duration of transgenerational inheritance in response to RNAi (72, 103).

Inherited silencing of genes expressed in somatic cells in *C. elegans*, which typically lasts for precisely one generation ((42), Fig. 1-3), could simply reflect silencing triggered by dsRNA in progeny without engaging any transgenerational gene silencing machinery within the parent germline. Similar direct delivery to progeny could underlie parental RNAi in insects (reviewed in ref. (27)), when dsRNA is delivered into the hemocoel (e.g., (104)) or through ingestion (e.g., (105)) to initiate RNAi. Additional studies are required to discover the evolutionarily-selected function, if any, for the delivery of ingested material – including regulatory RNA – directly into progeny.

5.3 RNAs in circulation as carriers of gene-specific information between generations

5.3.1 Inheritance of RNA as a mechanism to rapidly adapt to the environment

Molecules that can cross generational boundaries can cause apparent intergenerational effects. Exposure to some chemicals can cause multigenerational effects in mammals (e.g., endocrine disruptors (106) or odors (107)). Examination of how many generations the molecules used to trigger a response persist within an animal could inform mechanisms underlying multigenerational effects. Alternatively, intergenerational effects could result if RNAs carry sequence-specific information to gametes through circulation from distant tissues that experience chemicals, changes

in diet, or stress. In support of this possibility, studies focused on intergenerational and transgenerational effects in mammals implicate RNA in the inheritance of gene expression states across generations (108), report changes in small RNAs in gametes (109, 110), and report changes in RNAs acquired during gamete maturation from surrounding epithelia (22, 23). However, in all these cases, direct effects of a treatment (e.g., diet) on gametes and surrounding support tissues that alter RNA composition in gametes have not been ruled out. Furthermore, while extracellular RNAs have been detected in mammals, their biology is not well understood and is under intense investigation (see ref. (111) for a recent review). Using genetic mutants and fluorescently labeled RNAs to control and follow the traffic of extracellular RNAs, our results demonstrate their direct transfer between generations in an animal – an inheritance that can potentially vary based on parental experience.

The transport of RNA from parent to progeny in response to an ancestral experience may be a mechanism for organisms to rapidly adapt to conditions experienced in the environment. Perhaps these mechanisms exist to equip progeny for survival in adverse environmental conditions.

5.3.2 Inheritance of RNA from the environment as a host-pathogen response

Some RNAs can be transferred from one organism to another. RNA from *Wolbachia* bacteria can be detected in *Aedes aegypti*, *Drosophila melanogaster*, and *Drosophila simulans* (112). Small RNAs derived from dsRNA in corn and tomato plants can be detected in western corn rootworms and colorado potato beetles, respectively (113). The transfer of RNA from rice into humans (114) and from bacteria into *C. elegans* (115) has also been reported, but has not been replicated by

other groups. Many invertebrate organisms such as arthropods (e.g., honeybees and ticks), planaria, cnidarians, and some nematodes and lepidopterans can also ingest naked dsRNA or bacteria that express dsRNA in the environment and this ingested dsRNA can silence specific genes in what is known as environmental RNAi (reviewed in (116)). In light of our observations that dsRNA in circulation can itself reach progeny, it is possible that these RNAs from other organisms can also reach progeny. Further studies are required to determine if any RNA from other organisms reach progeny from parents.

It is unknown why organisms have the ability to ingest exogenous RNA from the environment. It is possible that organisms have evolved this ability to send information about the environment to progeny (as suggested above) but this also allows pathogens to send RNA into a host organism to silence essential genes. For example, fungal RNAs can suppress plant immunity genes (117). To this end, RNAs from pathogens may also be transported from parents to progeny and silence genes in progeny, further extending the reach of pathogens to subsequent generations.

5.4 Implications for endogenous RNAs in the zygote

The observation that dsRNA from circulation can reach the 1-cell embryo raises the possibility that endogenous dsRNA expressed from outside the germline may also be present in embryos. Endogenous dsRNA are mostly derived from inverted repeat structures, bidirectional transcription, and antisense transcripts in the genome (118). In *C. elegans*, dsRNA producing regions were identified across the genome with enrichment in long introns and 3' UTRs (119). Some identified

endogenous dsRNA can regulate genes within cells (e.g., siRNAs from endogenous dsRNA can regulate genes in mouse oocytes (118)). If these endogenous dsRNA can reach circulation, they could potentially reach the zygote. Directly analyzing the extracellular fluid is difficult, particularly in smaller organisms, because it is technically challenging to collect the fluid without penetrating surrounding tissues. However, using our work as a guide we can begin to identify endogenous dsRNA from circulation that is present in the 1-cell embryo by comparing RNA species in different genetic mutant embryos (e.g., wild type vs *rme-2(-)*).

5.5 Vitellogenin as a carrier of experience across generations

Extracellular RNA depends on vitellogenin-mediated endocytosis in oocytes to reach progeny (Chapter 3), raising the possibility that extracellular RNA may reach progeny in a similar way in other organisms because of the similarities in vitellogenin protein structure, production, and transport (see Chapter 1 for background information on vitellogenin).

5.5.1 Vitellogenin can bind diverse molecules

In addition to binding lipids, vitellogenins can also bind a variety of other molecules including nucleic acids, bacteria, fungi, and metals. Purified vitellogenin from *C. elegans*, chicken, and frogs can bind DNA *in vitro* (120). RNA can be isolated with vitellogenin particles from *Xenopus* (121). In this dissertation, we propose that extracellular dsRNA may bind vitellogenin in *C. elegans*. FITC-LPS, Bodipy-cholesterol, and Lucifer Yellow can also adhere to vitellogenin granules in *C. elegans* (88, 122). Purified vitellogenin from fish and honeybees can bind bacterial

LPS and peptidoglycan components (123, 124). Vitellogenin from fish can bind surface glucan from fungi (124) and vitellogenin in frogs can bind metals like Zn (125). Because of the ability of vitellogenin to bind many things and to be transported to progeny, vitellogenin may function as a carrier of information in addition to its role as a nutrient source for the developing embryo. It will be essential to further define what binds vitellogenin within an organism to identify potential information that can be carried to progeny by vitellogenin.

5.5.2 Vitellogenin production can be regulated by the environment

Vitellogenin production can be influenced by environmental factors. In some mosquitoes, vitellogenin production depends on blood meal-associated signals (reviewed in (126)). In fish, estrogen from the environment can stimulate vitellogenin production in males where vitellogenin is typically not expressed, resulting in the accumulation of vitellogenin in the male testes (reviewed in (127)). These environmental effects alter vitellogenin production, which may then alter the amount of vitellogenin and its components (e.g., extracellular dsRNA) deposited in progeny. The amount and type of vitellogenin-associated components may be altered as a result of environmental influences (e.g., extracellular dsRNA may bind newly produced vitellogenin in males and be deposited to progeny).

5.5.3 Vitellogenin can regulate behavior

Vitellogenin can regulate behavior in some organisms. In the honeybee *Apis mellifera*, the female worker bees begin adulthood by nursing and caring for the young and then transition to foraging. Vitellogenin interacts with juvenile hormone to play a role in the transition from nursing to foraging behavior (reviewed in (128,

129)). Vitellogenin-associated factors, like extracellular dsRNA, may also be involved in these kinds of regulatory mechanisms.

5.5.4 Vitellogenin can carry information from parental circulation to progeny

In this dissertation, we propose that vitellogenin can carry information, like extracellular dsRNA, from parental circulation to progeny. Another example of vitellogenin carrying information to progeny is in honeybees. In honeybees, vitellogenin can bind pieces of bacteria in circulation and transport the bacterial fragments to progeny (123). Foraging bees bring bacteria-containing pollen to the hive to make food for the queen. Upon ingestion of the food by the queen, parts of digested bacteria reach circulation and can bind vitellogenin. Vitellogenin bound to fragments of bacteria is then deposited into oocytes and acts as an immune-priming signal to protect the colony from future bacterial infections.

5.5.5 The vitellogenin homolog apoB-100 may carry information in mammals

In mammals, the placenta is thought to have replaced the need for vitellogenin to nourish the developing embryo. Mammals do not express vitellogenin genes but do express apoB-100, the major protein component of mammalian LDL particles (60, 61). The apoB-100 protein is closely related to vitellogenin and is thought to be a descendant of vitellogenin. Chickens express both vitellogenin and apoB-100 and the same receptor on oocytes recognizes both proteins to induce receptor-mediated endocytosis (130).

The apoB-100 protein may carry information from parental circulation to progeny in mammals as vitellogenin does in other species. In mammals, miRNA bound to apoB-100 can be detected in circulation (131), granulosa cells can secrete

apoB-100 into follicular fluid (132), and the LDL-receptor for apoB-100 is present on developing oocytes (133). Interestingly, the mammalian SID-1 homolog enhances uptake of apoB-100 bound siRNA in hepatocytes (134). Perhaps apoB-100 carries information, like extracellular RNAs in circulation, to progeny and the mammalian SID-1 homolog enables entry of RNA into cells to regulate specific genes in progeny. The relevance of mechanisms elucidated in this dissertation to mammals could be worth exploring.

Chapter 6: Future directions

6.1 Preface

All data in this chapter was generated by Julia Marré.

Some worm strains were obtained from the *Caenorhabditis elegans* Genetic stock Center and the Seydoux laboratory (Johns Hopkins University). The Hamza laboratory (University of Maryland) provided some of the bacteria that express dsRNA. The Andrews laboratory (University of Maryland) trained me and allowed me to use their confocal microscope.

6.2 Introduction

In this dissertation, we propose a model where extracellular dsRNA is transported through oocytes held within vitellogenin-containing vesicles to progeny where silencing of genes can occur as early as the 16-cell embryo. During early embryogenesis, inherited dsRNA and vitellogenin appear to separate but then accumulate in gut cells over time.

In this chapter, future directions to expand on these observations will be discussed and preliminary work already performed to achieve these future directions will be detailed. Specifically, we propose essential next steps include identifying the location of SID-1 within the germline and embryos, interrogating the relationship between vitellogenin and dsRNA, and finding endogenous and/or exogenous RNA from circulation that can reach progeny.

6.3 Materials and methods

6.3.1 Strains, transgenesis, and oligonucleotides

All strains used are listed in Table 6-1 and all oligonucleotides used are listed in Table 6-2. Strains were cultured and maintained as described in (70).

Table 6-1. Strains used.

Strain	Genotype
AMJ327	<i>cclIs4251 I</i> ; <i>sid-1(qt9) V</i> ; <i>jamIs2 [Pmyo-3::SID-1cDNA::DsRed]</i>
AMJ576	<i>jamSi12[Pmex-5::sid-1::DsRed::sid-1 3' UTR] II</i> ; <i>unc-119(ed3) III</i> ; <i>sid-1(qt9) V</i>
AMJ587	<i>mut-2(jam9) I</i>
AMJ609	<i>mut-2(jam9) I</i> ; <i>jamSi12[Pmex-5::sid-1::DsRed::sid-1 3' UTR] II</i> ; <i>unc-119(ed3) III</i> ; <i>sid-1(qt9) V</i>
AMJ650	<i>sid-1 (qt9) V</i> ; <i>jamEx189[Pmyo-3::sid-1::DsRed::sid-1 3' UTR]</i>
AMJ706	<i>sid-1(qt9) V</i> ; <i>jamEx193[Pmyo-3::sid-1::gfp]</i>
HC195	<i>nrIs20 [Psur-5::sur-5::gfp::unc-54 3' UTR] IV</i>
HC196	<i>sid-1(qt9) V</i>
N2	wild type
TX189	<i>unc-199(ed3) III</i> ; <i>teIs1[Poma-1::oma-1::gfp]</i>
TX691	<i>unc-199(ed3) III</i> ; <i>teIs46[Pend-1::gfp]</i>

Table 6-2 Oligonucleotides used (5' to 3', IDT).

Oligonucleotide	Sequence
P1 (<i>Pmex-5_fwd</i>)	ggtaccctctagtcaggcctatagaaaagttgaaatatcagtttta aaaa
P2 (<i>Pmex-5_rev</i>)	cacgaatcattctctgtctgaaacattcaattg
P3 (<i>sid-1_fwd</i>)	cagacagagaatgattcgtgtttatttgataattttaatg
P5 (<i>DsRed_fwd</i>)	taacattttcatggcctcctccgagaac
P6 (<i>DsRed_rev</i>)	aattactctactacaggaacaggtggtgg
P7 (<i>sid-13'UTR_fwd</i>)	gttctgtagtagagtaattttgtttccctatc
P8 (<i>sid-1 3'UTR_rev</i>)	ggctacgtaatacgaactcacagtggctgaaaattatgc
P9 (Mos_insertion_1)	ataaggagttccacgcccag
P10 (Mos_insertion_2)	ctagtgagtcgtattataagt
P11 (Mos_insertion_3)	tgaagacgacgagccacttg
P12 (<i>mut-2</i> genotype_fwd)	catttgatgcatttcctcca
P13 (<i>mut-2</i> genotype_rev)	atgcttgtagaaatccgggta
P14 (<i>Pmyo-3_fwd</i>)	tatagtcctgtcgggtttcg
P15 (<i>Pmyo-3_rev</i>)	aattatcaaataaacacgaatcatttctagatggatctagtggtc
P16 (<i>Pmyo-3sid-1_Fwd</i>)	gaccactagatccatctagaaatgattcgtgtttatttgataatt
P17 (<i>sid-1 3'UTR_rev</i>)	tcatgcacaacaaagccgac
P18 (RT_ <i>gfp</i> sense)	agggcagattgtgtggacag
P19 (RT_ <i>gfp</i> antisense)	aactacctgttccatggcca
P20 (PCR_ <i>gfp</i> fwd)	aagagtgccatgcccgaag

P21 (PCR_ <i>gfp</i> rev)	ccatcgccaattggagtagtt
P22 (RT_ <i>tbb-2</i> sense)	tcgtcttcggcagttgcttc
P23 (PCR_ <i>tbb-2</i> fwd)	gacgagcaaatgctcaacg
P24 (PCR_ <i>tbb-2</i> rev)	ttcggatgaactccatctcg
P25 (nestedPCR_ <i>tbb-2</i> fwd)	tcccgaacaacgtcaagacc
P26 (nestedPCR_ <i>tbb-2</i> rev)	cccaatggaggaaagccttgc
P27 (<i>gfp</i> sense RNA)	ugguccuucuugaguuuguaacagcugcugggauuacaca uggcauggau
P28 (5' Atto 565 labeled <i>gfp</i> antisense ssRNA)	5'Atto 565-auccaugccauguguaauccagcagcuguu acaacucaagaaggacca

To express SID-1::GFP in the muscle (*sid-1(-); Ex[Pmyo-3::sid-1(+):gfp]*):
 HC196 hermaphrodites were injected with 10 ng/μl pTK2 (*Pmyo-3::sid-1(+):gfp*; made by Tessa Kaplan in the Hunter Lab) and progeny expressing green fluorescence were isolated (AMJ706). Animals were fed *unc-22* RNAi to confirm rescue of SID-1 in the muscle.

To express SID-1 in the germline (*Si[Pmex-5::sid-1(+):DsRed::sid-1 3'utr]*):
 The promoter for *mex-5* (*Pmex-5*) was amplified (Phusion polymerase, NEB) from pJA252 (addgene #21512) using the primers P1 and P2. The *sid-1* gene was amplified (Phusion polymerase, NEB) from N2 genomic DNA using the primers P3 and P4. The *DsRed* gene was amplified (Phusion polymerase, NEB) from pAJ53a (*Pmyo-3::sid-1(+):DsRed::unc-54 3'utr*; made by Tessa Kaplan in the Hunter Lab) using the primers P5 and P6. The *sid-1 3' utr* was amplified (Phusion polymerase, NEB)

using the primers P7 and P8. Using Gibson Assembly Mastermix (NEB), these four amplicons were placed into pCFJ151 (addgene #19330) digested with AflII (NEB) and treated with CIP (NEB) to generate pJM10. pJM10 (50 ng/μl) and the coinjection markers pCFJ601 (50 ng/μl), pMA122 (10 ng/μl), pGH8 (10 ng/μl), pCFJ90 (2.5 ng/μl), and pCFJ104 (5 ng/μl) (plasmids described in (71)) were injected into the germline of adult EG4322 animals. One transgenic line was isolated as described earlier (50) and crossed into *sid-1(qt9)* animals to generate AMJ576. The integration of *Pmex-5::sid-1(+)::DsRed::sid-1 3'utr* in AMJ576 was verified by genotyping the complete sequence of the insertion. AMJ576 hermaphrodites were crossed with AMJ587 males and F2 progeny were genotyped for *Pmex-5::sid-1(+)::DsRed::sid-1 3'utr* using primers P9, P10, and P11 and for *mut-2(jam9)* using primer P12 and P13 to generate AMJ609.

To express SID-1::DsRed in the muscle (*sid-1(-); Ex[Pmyo-3::sid-1(+)::DsRed::sid-1 3'utr]*): The promoter for *myo-3* (*Pmyo-3*) was amplified (Phusion polymerase, NEB) from pCFJ104 (addgene #19328) using the primers P14 and P15. The *sid-1(+)::DsRed::sid-1 3' UTR* construct was amplified (Phusion polymerase, NEB) from pJM10 (*Pmex-5::sid-1(+)::DsRed::sid-1 3'UTR* in pCFJ151) with primers P16 and P17. The final PCR products were purified (QIAquick PCR Purification Kit, Qiagen) and injected into HC196 hermaphrodites at 10 ng/μl. Progeny expressing DsRed were isolated (AMJ650) and fed *unc-22* RNAi to confirm rescue of SID-1 in the muscle.

6.3.2 P0 + F1 feeding RNAi

RNAi bacteria was grown in LB-carbenicillin overnight and 100 µl was seeded on RNAi plates (NG agar plate supplemented with 1mM IPTG (Omega) and 25 µg/ml carbenicillin (MP Biochemicals)). L4-staged animals were added to seeded RNAi plates and incubated at 20°C for 4 days. On the fourth day, F1 progeny were assessed for silencing phenotype.

Table 6-3. Scoring of gene-specific silencing.

Gene	Site expressed	Defect scored upon RNAi
<i>pos-1</i>	Germline	Dead (unhatched) eggs
<i>unc-22</i>	body-wall muscle	L4 or young adults continuously twitch within 1 minute in response to 3mM levamisole (Sigma Aldrich).

6.3.3 Fixing and sorting embryos

Preliminary method for embryo isolation and methanol fixation (an adaptation of methods in (135)): Two L4-staged worms were placed on 25 NG plates seeded with OP50 and incubated at 20°C for 5 days. Plates were then washed with cold M9 buffer using a glass pipette to scrape the agar to release laid embryos. Worms/embryos in buffer were then placed in a 1.5 mL microcentrifuge tube and spun at 310 g for 30 s. The supernatant above live worms/embryos was decanted leaving a pellet of ~300 µl of worms/embryos in M9 buffer. 8.25% NaOCl (concentrated Clorox bleach) was added to the pellet and let sit on ice for ~11

minutes. Cultures were then spun at 310 g for 30 s and supernatant was removed. Cold M9 buffer was added, tubes were flicked to mix, and then spun at 310 g for 30 s. The supernatant was removed and cold M9 buffer was added again, tubes flicked, and spun at 310 g for 30 s. After the second wash, supernatant was removed again and 1 mL cold M9 buffer was added and mixture was put through a 40 μ m nylon mesh cell strainer (Falcon) into a 50 mL conical tube. Another 1 mL of cold M9 buffer was added on top of the cell strainer in an attempt to ensure all embryos had been passed through the strainer. Upon viewing the cell strainer after straining, embryos could be seen stuck on top of the cell strainer (i.e., did not pass through and will not be part of the sample that is kept as the flow-through) and embryos could also be found in the flow-through. The flow-through liquid was then placed in a 1.5 mL microcentrifuge tube and spun at 310 g for 30 s. The supernatant was removed leaving a pellet of ~200 μ l. 800 μ l of 100% methanol was added and tubes were rotated on a nutator for 1.5 hours at 4°C. Tubes were then spun at 300 g for 1 minute, the supernatant was decanted, cold cell culture grade PBS was added to the pellet, and tubes were kept on ice.

Sorting embryos: Fixed embryos were sorted in the large particle sorter COPAS FP (Union Biometrica). Events were gated to capture embryo sized particles and any particle with fluorescence with the 488 nm laser. Sorted events were placed on the lid of a petri dish to be imaged for accuracy.

6.3.4 Embryo RT-PCR

To collect embryos: A 5 mm round coverslip (VWR) was placed on top of 0.75 μ l of RNase- and DNase-free water (IDT) on a microscope slide (VWR). (Water

is needed to hold the coverslip in place.) 4 μ l of RNase- and DNase-free water (IDT) was added on top of the coverslip and 3 adult worms were placed in the water. Adult worms were cut in the middle of the worm with a scalpel to release embryos from the uterus. Large worm remnants were picked out of the water and then most of the water was removed from the coverslip leaving embryos in a small volume. 5 μ l of bleach solution (80 mM KOH, 50 mM NaOCl) was added to the embryos and incubated at room temperature for 30 seconds. About 5 μ l of this solution was removed and the embryos were subjected to a series of washes. The first wash was with 10 μ l of 10 mg/ml BSA (NEB). This was followed by two washes with 5 μ l of RNase- and DNase-free water (IDT) where the water were pipetted up and down. The embryos were then moved to a new coverslip and the water washes were repeated. The washed embryos were suspended in 2 μ l of water and 0.5 μ l of this water was removed from the suspended embryos and placed on a separate coverslip to be processed as a negative water control to test for residual extracellular RNA. A new coverslip was placed gently on top of the embryos, the number of embryos between the coverslips were counted, and then pressure was applied to squish the embryos. Squished embryos and coverslips were placed into 1.5 ml microcentrifuge tubes (VWR) and flash frozen in liquid nitrogen.

Reverse transcription (based on (136)): Coverslips with the squished embryos were crushed with a 1.5mL pestle (VWR). 50 μ l of RT mastermix (1x first strand buffer (Roche), 0.1 M DTT (Roche), 0.5% (vol/vol) Triton-X (EMD), 100 μ g/mL BSA (NEB), 0.4 units/ μ l SUPERase In RNase inhibitor (Life technologies), 10mM dNTP (NEB), Rnase/DNase free water (IDT)) was added to the crushed material

while keeping the pestle within the microcentrifuge tube. The pestle was removed and tubes were briefly vortexed and spun. The mixture was separated into six 200 µl PCR tubes (VWR) at 4 µl per tube. 0.5 µl of RT primer P18, P19, or P22 (at 2pM) was added before samples were heated at 95°C for 5 minutes. Samples were then chilled on ice for 3 minutes before adding either 0.5 µl Superscript III Reverse transcriptase (Roche) or RNase/DNase free water (IDT). Samples were incubated in a thermal cycler (eppendorf) at 55°C for 60 minutes and then inactivated at 70°C for 15 minutes.

PCR: 45 µl of PCR mastermix (5 µl 10X PCR buffer, 0.5 µl 10 mM dNTP mix (NEB), 1 µl 10 µM fwd and rev primer (P20 and P21 or P23 and P24), 0.5 µl Taq polymerase, 37 µl water) was added to each RT sample and incubated in a thermal cycler for 35 cycles with an annealing temperature of 55°C. For *tbb-2*, this PCR product was then diluted 1:100 and then 5 µl was added to 45 µl of PCR mastermix with P25 and P26 and incubated in a thermal cycler for 35 cycles with an annealing temperature of 55°C. PCR products were run in 2% agarose gels, stained with ethidium bromide (amresco), and then imaged with the Molecular Imager Gel Doc XR (Bio-Rad) using ultraviolet light.

6.3.5 Soaking RNAi

Fluorescently labeled *gfp*-dsRNA was made as described in Chapter 3.3.3 annealing P27 and P28.

Gravid adults 24 hours post L4-staged animals were placed in 6 µl of ~185.29 ng/µl Atto 565-labeled *gfp*-dsRNA (see Fig. 3-1 for details) for differing amounts of

time (3, 5, or 18 hours) before animals were imaged. For worms in the 18 hour soak in dsRNA, 10 µl of liquid OP50 culture was added after 5.5 hours.

6.3.6 Fluorescence imaging

All images were adjusted for display using ImageJ (NIH).

Transgenic lines expressing *sid-1(+):DsRed::sid-1 3'utr*: L4 animals were placed in 3 mM tetramisole hydrochloride (Sigma) and imaged using the Eclipse Ti Spinning Disk Confocal (Nikon) with the 100X objective lens. DsRed was excited using a 561 nm laser and fluorescence was collected through a 415-475 nm and 580-650 nm emission filter. GFP was excited using a 488 nm laser and fluorescence was collected through a 500-550 nm emission filter.

Embryo fixing and sort: Embryos post fixation and post sort were placed on the lid of an empty petri dish in a droplet and imaged at fixed magnification on an AZ100 microscope (Nikon) with a Cool SNAP HQ² camera (Photometrics). A C-HGFI Intensilight Hg Illuminator was used to excite GFP (filter cube: 450-490 nm excitation, 495 dichroic, and 500-550 nm emission). Exposure times were scaled to just under saturation for each genetic background. Corresponding bright-field images were taken using auto-exposure.

Soaking RNAi: Gravid adult animals were placed in 3mM tetramisole hydrochloride (Sigma) and imaged using the Eclipse Ti Spinning Disk Confocal (Nikon) with the 60X objective lens. Atto 565 was excited using a 561 nm laser and fluorescence was collected through a 415-475 nm and 580-650 nm emission filter. GFP was excited using a 488 nm laser and fluorescence was collected through a 500-550 nm emission filter.

6.4 Where does SID-1 localize in the germline and in embryos?

6.4.1 Introduction

We have observed the inheritance of extracellular dsRNA to progeny without entering the cytosol of parental cells (Chapter 2 and 3) and silencing in progeny was observed as early as the 16-cell embryo (Fig. 4-2 B), suggesting that inherited dsRNA can enter the cytosol before this stage. To determine when and where inherited dsRNA can enter the cytosol through the dsRNA importer SID-1, the location of SID-1 in the germline, the oocytes and the embryo must be identified.

mRNA-seq and *in situ* analyses indicate *sid-1* is most prominently expressed in the germline and that there is maternal deposition of *sid-1* mRNA transcripts to pre-transcriptional embryos (95-98). Using translational multicopy and extrachromosomal reporters, SID-1 has been proposed to localize to the cell membrane in somatic cells in *C. elegans* (38). However, multicopy arrays are silenced in the germline thus requiring further analyses with single-copy transgenes or tagging of the endogenous *sid-1* gene to determine the localization of SID-1.

6.4.2. Visualizing SID-1 in the germline using single-copy transgenes

To visualize SID-1 in the germline, single-copy transgenes or direct editing of the endogenous *sid-1* locus must be performed. Single-copy transgenes can be made by inserting a promoter::gene construct into specific loci in the *C. elegans* genome containing the *Drosophila Mos1* transposon, or *Mos1*-mediated Single Copy Insertion (71). Excision of the *Mos1* transposon creates a dsDNA break that can be repaired using the homologous sequences present on either side of the promoter::gene construct. Using MosSCI, we made three independent lines expressing *DsRed*-tagged

sid-1 under the germline-specific *mex-5* promoter with the native *sid-1* 3' *utr* (*Pmex-5::sid-1::DsRed::sid-1* 3' *utr*, Figure 6-1 A). However, we could neither detect any fluorescence in any tissue in the worm (data not shown) nor rescue the *sid-1*(-) phenotype (Fig. 6-1 B). Concerned that the *sid-1::DsRed::sid-1* 3' *utr* contained a mutation, we sequenced the entire transgene and generated a strain that expresses *sid-1::DsRed::sid-1* 3' *utr* under a muscle promoter (*Pmyo-3*) from a multicopy transgene. We detected no mutations in the MosSCI transgene, were able to visualize SID-1::DsRed::SID-1 3' UTR in the muscle (data not shown), and rescued the *sid-1*(-) phenotype in muscles with SID-1::DsRed::SID-1 3' UTR (Fig. 6-1 C), suggesting that there are no mutations in the transgene and the transgene is able to produce functional SID-1::DsRed. Others have observed stochastic silencing of transgenes made using MosSCI in a process known as RNA-induced epigenetic silencing (RNAe) (137). RNAe of transgenes can be relieved in the absence of the putative nucleotidyl transferase MUT-2. However, upon placing the *Pmex-5::sid-1::DsRed::sid-1* 3' *utr* transgene into a *mut-2*(-) background, we were still unable to detect any fluorescence or rescue of *sid-1*(-) phenotype in the germline (Fig. 6-1 B), suggesting that RNAe may not be the cause of the lack of fluorescence. Further investigation is required to determine the cause of the lack of fluorescence of this transgene, including RT-PCR to interrogate mRNA levels.

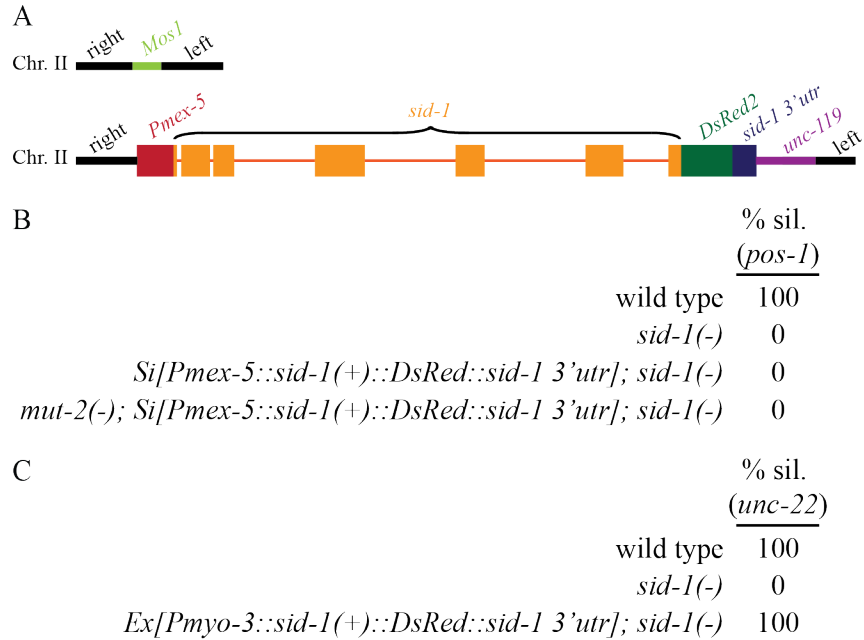


Fig. 6-1. Single-copy insertion of *sid-1* tagged with *DsRed* and expressed under a germline promoter does not express a functional SID-1::DsRed protein. (A) Schematic of the DNA inserted at Mos1 locus on chromosome II. germline promoter (*Pmex-5*), *sid-1* coding gene with introns, *DsRed2*, and *sid-1* 3'utr. (B) There is no detectable silencing of a germline gene (*pos-1*) upon feeding RNAi of worms with the *Pmex-5::SID-1::DsRed::sid-1* 3'utr single-copy insertion. (C) Silencing of a muscle gene (*unc-22*) is detectable when the DNA for *sid-1::DsRed::sid-1* 3'utr is expressed under a muscle promoter (*Pmyo-3*). n > 9 in (B) and (C).

6.4.3 SID-1 localizes to vesicular structures near the nucleus in muscle cells

To begin investigating the localization of SID-1 in the soma, we made transgenic worms expressing *DsRed*- or *gfp*- tagged *sid-1* in muscles in a *sid-1*(-) background. In contrast to the original paper of the discovery of SID-1 (38), functional *DsRed*- and *GFP*- tagged SID-1 localized to vesicular structures near the nucleus (Fig. 6-2) and not the cell membrane. Interestingly, the mammalian homolog of SID-1 was also reported to localize to intracellular vesicles (52). By comparing these vesicular structures containing SID-1 with some known compartments in muscles as observed by others using fluorescent markers, SID-1 does not appear to

localize to known compartments in muscles (Fig. 6-3). While SID-1 may be present in a unique compartment in muscles, only a subset of known compartments in muscles with fluorescent tags were compared, requiring further investigation to identify these vesicular structures. However, since *sid-1* is most prominently expressed in the germline (95-98), it is possible that endogenous SID-1 does not even localize in this pattern in wild-type animals.

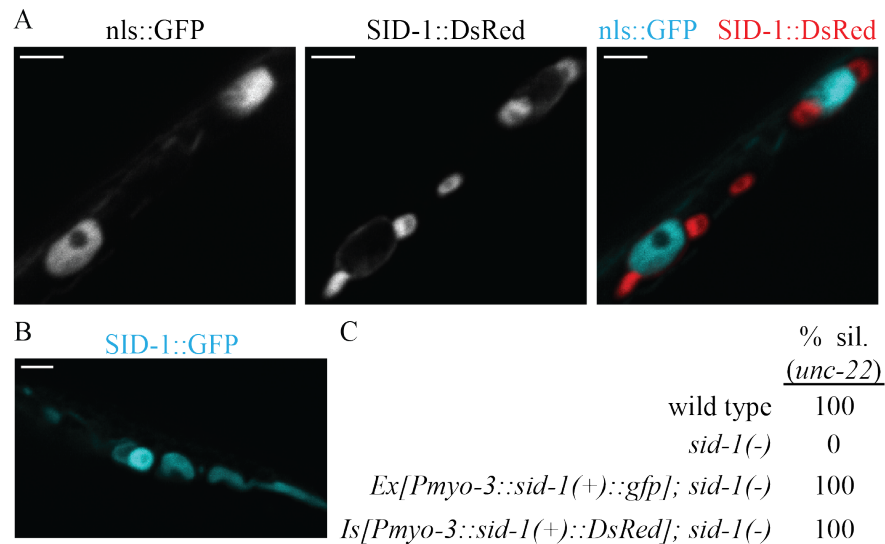


Fig. 6-2. Functional SID-1 localizes to vesicular structures in muscle cells. (*A* and *B*) Representative images of SID-1 tagged with DsRed (*A*) or GFP (*B*) in muscle cells. Nuclear localized GFP (nls::GFP) is also present in (*A*). Scale bars, 5 μ m (*C*) By silencing observed in a feeding RNAi experiment, SID-1 tagged with DsRed and GFP can be inferred to be functional rescues of *sid-1(-)* phenotype in muscles. $n > 34$ (*C*).

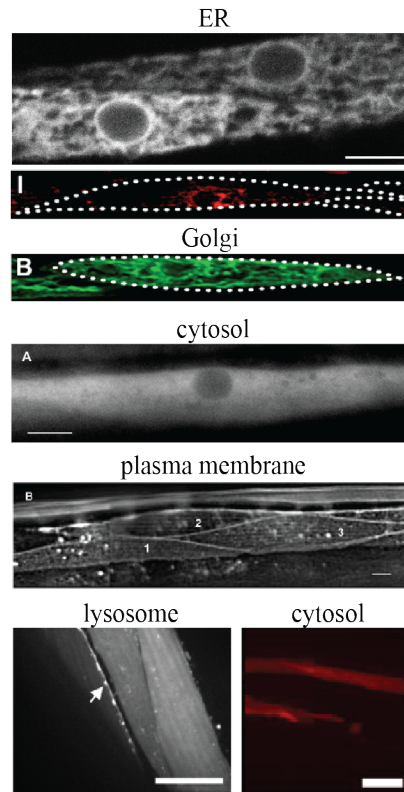


Fig. 6-3. The vesicular structures where SID-1 localizes in muscles do not look similar to known compartments in the muscle. Representative images of muscles in reporter strains expressing GFP or DsRed in different compartments of muscle cells. Images are from (138-141).

6.5 What mechanisms control the interaction between dsRNA and vitellogenin?

6.5.1 What properties enable vitellogenin and dsRNA to interact?

Extracellular dsRNA and vitellogenin can enter oocytes through RME-2-mediated endocytosis and are presumably held within the same intracellular vesicles as indicated by their colocalization in oocytes (Fig. 3-5 A). Extracellular dsRNA and vitellogenin were also observed to bind in lipid droplets outside the germline between embryos in the uterus (Fig. 3-7 B), suggesting that dsRNA and vitellogenin have the ability to bind each other in the extracellular space. To interrogate the requirements

for the association of vitellogenin and dsRNA, other fluorescently labeled nucleic acids should be tested for their interaction with vitellogenin. In particular, double stranded DNA, single stranded RNA, and DNA:RNA hybrids should be injected into the body cavity of worms expressing *vitellogenin::gfp* and injected animals should be imaged for colocalization in the germline and outside the germline (e.g., the vitellogenin droplets within the uterus).

6.5.2 What mechanisms control the trafficking of inherited dsRNA and vitellogenin in the embryo?

Inherited dsRNA and vitellogenin exist within the same intracellular vesicles in early embryos (Fig. 4-6). To determine the mechanisms that control the trafficking of dsRNA and vitellogenin in the embryo, it is necessary to extract and isolate intracellular vesicles and identify proteins and nucleic acids within these vesicles by performing RNA-seq and mass spectrometry experiments (using methods similar to those used to isolate and characterize extracellular vesicles from ciliated neurons (142)). Additionally, colocalization between inherited dsRNA and known membrane proteins should be assessed using worm strains that express fluorescently-tagged fusion proteins (e.g., early and late endosome and lysosome markers). These colocalization experiments can help identify known processes involved in dsRNA and vitellogenin trafficking in the embryo.

6.5.3 Does inherited dsRNA separate from vitellogenin in early development?

Inherited dsRNA and vitellogenin enter oocytes together and remain primarily colocalized in the 1-cell embryo, but then begin to separate by ~ 8-cell embryo (Fig. 4-6). This suggests that there are mechanisms in the embryo to separate inherited

vitellogenin from other inherited information. However, later in development, both inherited dsRNA and vitellogenin accumulate in gut cells in the embryo (Fig. 4-7 and Fig. 4-8), suggesting that the bulk of inherited dsRNA follows the fate of vitellogenin through development, inherited dsRNA separates from vitellogenin early in development and is then reunited with vitellogenin later in development, or a combination of both occurs. To distinguish between these possibilities, fixed embryos containing inherited dsRNA and vitellogenin should first be imaged to rule out the possibility that the separation of inherited dsRNA and vitellogenin in early development is due to live imaging conditions. Single embryos should also be imaged from fertilization through hatching to further define the location of inherited dsRNA and vitellogenin through the entirety of embryonic development.

6.6 What exogenous and endogenous extracellular RNAs are in the zygote and when can processing of inherited RNAs occur?

6.6.1 Introduction

Inherited extracellular dsRNA was capable of silencing genes of matching sequence in embryos as young as 16-cell staged (Fig. 4-2 *B*), suggesting that dsRNA can enter the cytosol and can be processed by this stage. Extracellular dsRNA did not need to enter the cytosol of any cell in parents to be inherited (Fig. 2-12 *B*, Fig. 3-8). Similarly, transgenic dsRNA expressed endogenously from within neurons can silence genes in progeny without entry into the cytosol of any parental cell (Fig. 2-12 *C*), suggesting that endogenously expressed dsRNA may reach the extracellular space and be transported to progeny as observed for exogenous dsRNA introduced into the

extracellular space. However, it is unclear what endogenous dsRNA is present in the extracellular space that can be inherited and when inherited dsRNA is processed in progeny. Because extracellular dsRNA must enter oocytes through RME-2-mediated endocytosis to reach progeny, RNA-seq should be performed on wild-type 1-cell embryos to determine endogenous dsRNA present in the extracellular space of parents. RNA present in wild-type but not in *rme-2(-)* embryos are likely candidates for endogenous RNA that can be inherited. Collecting wild-type embryos of different stages during early development (e.g., 1-cell, 8-cell) from parents exposed to dsRNA and performing RNA-seq and northern blotting at each stage will resolve when inherited RNA processing occurs and what RNA species are present at each stage of development.

6.6.2 Collecting large samples of embryos using sorting

To perform RNA analyses of inherited RNA in the embryo, it is essential to collect a lot of embryos at specific stages. Large populations of stage-specific embryos can be collected using fluorescent transgenes and a COPAS Biosorter. The COPAS Biosorter can sort based on particle size and/or fluorescence intensity. Using fluorescent transgenes that express during specific stages of the embryo, embryos can be sorted into different stage-specific populations. We will use three different fluorescent transgenes to sort 1-cell (*Poma-1::oma-1::gfp*), 8-cell (*Pend-1::gfp*), and > 8-cell (*Psur-5::sur-5::gfp*) staged embryos.

We began by sorting embryos expressing the brightest GFP, *Psur-5::sur-5::gfp*, by gating for the smallest size events that also contained GFP fluorescence. Of the nine events called by the COPAS Biosorter, eight were true events or embryos

expressing GFP (Fig. 6-4), suggesting that sorting embryos with a bright GFP is feasible.

The 1-cell embryo becomes a 2-cell embryo in ~40 minutes (143). Therefore, it is necessary to fix embryos before sorting to prevent the maturation of all 1-cell embryos before processing is complete. We fixed *Poma-1::oma-1::gfp* and *Pend-1::gfp* embryos using a method adapted from (135). In contrast to (135), we observed altered embryo morphology and fluorescence in *Poma-1::oma-1::gfp* embryos post fixing (Fig. 6-5), suggesting that the fixing method is damaging embryos. We did not image *Pend-1::gfp* embryos post fixing. We sorted these fixed embryos using the same gating conditions as *Psur-5::sur-5::gfp* and collected 3/8 and 7/10 true events out of total events for *Poma-1::oma-1::gfp* and *Pend-1::gfp*, respectively (Fig. 6-6). These results suggest that the fixing method needs to be optimized so that embryos are not damaged. The variation in the rate of true events out of total events may be due to the inability of the COPAS Biosorter to correctly detect the fluorescence intensity of these dimmer GFP from *Poma-1::oma-1::gfp* and *Pend-1::gfp*. Therefore, after optimizing the fixation method, sorting should be assessed and replicated to determine the accuracy of sorting before subsequent experiments are attempted.

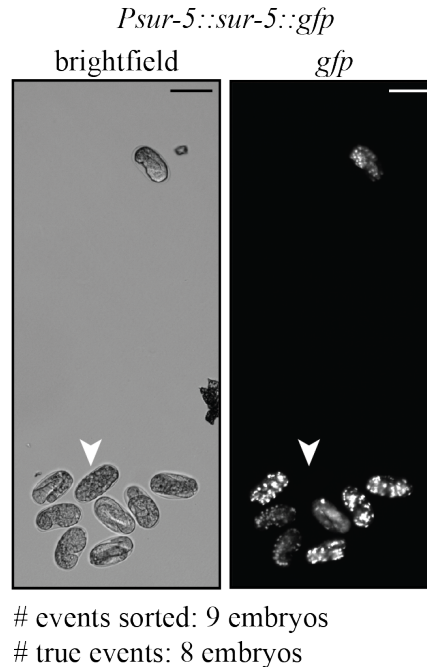


Fig. 6-4. Embryos expressing a bright *gfp* gene can be sorted. Embryos expressing *Psur-5::sur-5::gfp* (expressed most prominently in gut cells) were sorted. 9 events were sorted but of the 9 events, only 8 were true events (embryos with green fluorescence). False events are indicated by arrowheads. Scale bars, 50 μ m.

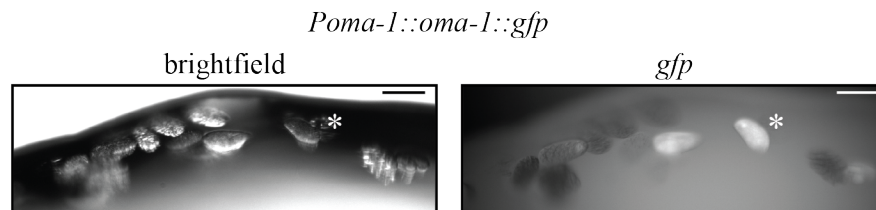


Fig. 6-5. Embryo morphology and *gfp* fluorescence appear altered post bleach and methanol fixation. Embryos expressing *Poma-1::oma-1::gfp* (expressed in 1 and 2-cell embryos) were bleached from gravid adults and then fixed with methanol. Asterisk indicates an embryo that appears to be a 2-cell embryo in the *gfp* channel (*right*) but looks more developed in the brightfield channel (*left*). Embryos are at the edge of a water droplet. Scale bars, 50 μ m.

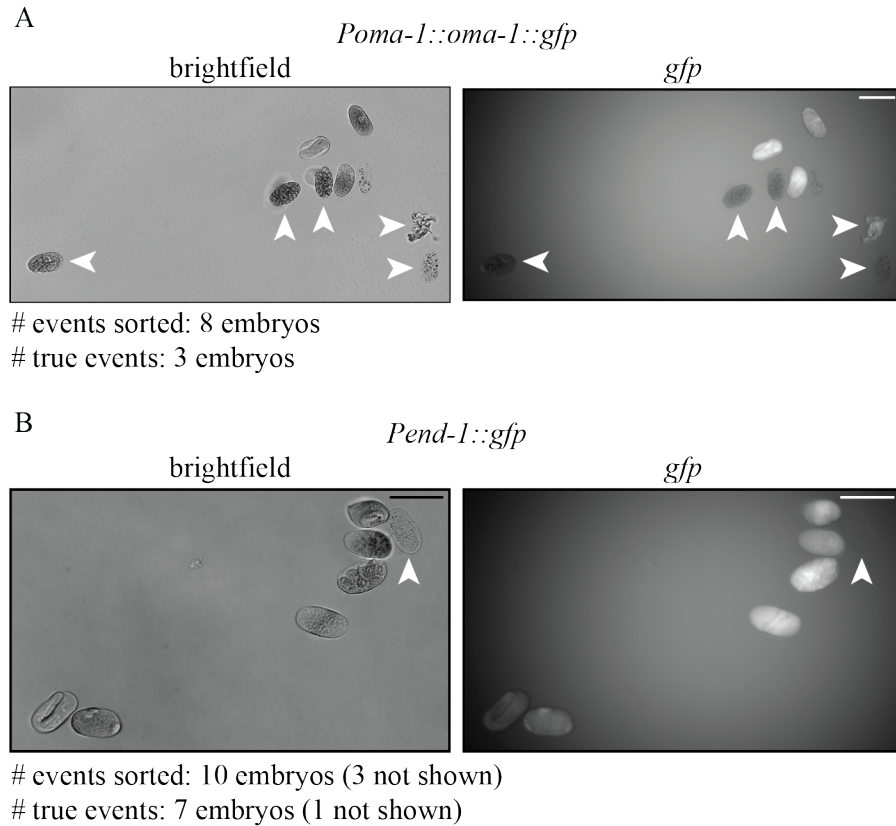


Fig. 6-6. Embryos expressing a *gfp* gene that produces dim fluorescence can be sorted but there is an increase in false events. (A) Embryos expressing *Poma-1::oma-1::gfp* (expressed in 1 and 2-cell embryos) were sorted and only 3 out of 8 events were true events or embryos with green fluorescence. Exposure time, 4.7s. (B) Embryos expressing *Pend-1::gfp* (expressed in early gut cells) were sorted and 7 out of 10 events were true events (embryos with green fluorescence). Exposure time, 6.7s. False events are indicated by arrowheads. Scale bars, 50 μ m. Note that for the embryos containing fluorescence, we do not know if this fluorescence is due to GFP or autofluorescence.

6.6.3 Detecting inherited RNA in embryos using RT-PCR

RT-PCR can be used to detect inherited RNA species in embryos. To detect differences in inherited RNA between single embryos, we attempted to collect single embryos from adults fed *gfp* RNAi and perform RT-PCR for inherited long *gfp*-dsRNA using sequence specific RT and PCR primers (Fig. 6-7 A). Using sequence specific RT and PCR primers, we could specifically detect sense and antisense strands from *in vitro* transcribed *gfp*-dsRNA (Fig. 6-7 B). Next, we collected a small number of embryos from adult worms by cutting the worm near the vulva using a scalpel, removing the adult worm carcass, and then treating the embryos released from the adult with bleach and washing embryos to destroy any nucleic acids present outside embryos. To optimize the embryo collection and RT-PCR conditions, we began by collecting 3 embryos and 16 embryos and then used primers specific to *tbb-2*, a gene that is highly expressed in the germline and present in embryos (144, 145). We could detect spliced *tbb-2* RNA in embryos and not in the water surrounding embryos (Fig. 6-8). However, we also detected DNA in the water surrounding embryos (Fig. 6-8, band in + and – lanes for water surrounding embryo lanes), suggesting that the method used to bleach and wash embryos is capable of eliminating RNA but not DNA surrounding embryos. Increasing the length of time embryos are exposed to bleach, the concentration of bleach, or the number of washes performed on isolated embryos may eliminate DNA. Since the ultimate goal is to detect dsRNA, which is more stable than ssRNA like *tbb-2* mRNA, it is essential to optimize this step before continuing with subsequent experiments.

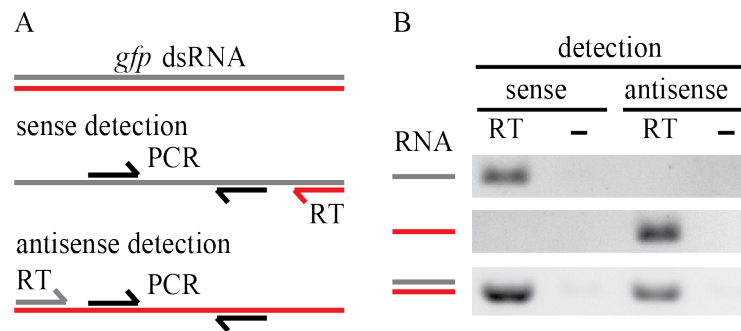


Fig. 6-7. *gfp*-dsRNA can be detected by RT-PCR using sequence specific RT and PCR primers. (A) Schematic of sequence specific primers to detect sense and antisense strands of *gfp*-dsRNA. (B) RT-PCR of single stranded sense (grey), single stranded antisense (red) and double stranded (grey and red) RNA using sense and antisense specific RT primers. Samples run in 2% agarose gel and stained with EtBr.

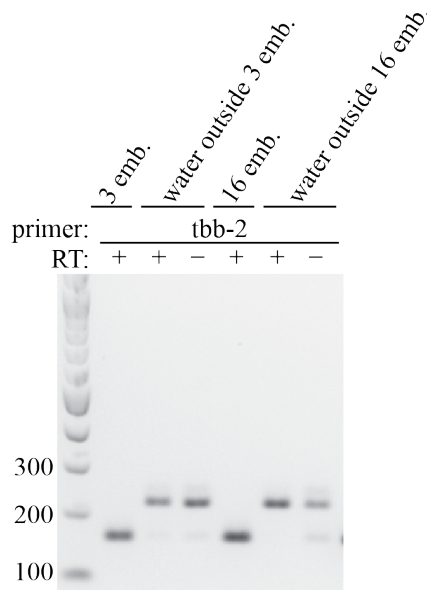


Fig. 6-8. The bleaching of gravid adults to isolate early embryos does not eliminate contaminating maternal DNA surrounding the embryos. RT-PCR using *tbb-2* sequence specific RT primer, initial PCR and then nested PCR of embryos (emb.) or water surrounding embryos post bleaching method. Samples run in 2% agarose gel with expected band sizes of 212 bp for unspliced *tbb-2* DNA or cDNA and 157 bp for spliced *tbb-2* cDNA.

6.6.4 Introducing exogenous dsRNA by soaking RNAi

Exogenous dsRNA introduced to parent worms can silence specific genes in progeny (34). Exogenous dsRNA can be introduced to worms by allowing worms to ingest bacteria that express dsRNA (feeding RNAi), by injecting worms with *in vitro* transcribed dsRNA outside the germline, or by soaking worms with *in vitro* transcribed dsRNA (soaking RNAi) (35). While dsRNA is directly placed outside the germline when injecting into the body cavity, dsRNA may first enter the gut and then enter the body cavity when dsRNA is introduced by soaking or feeding RNAi. While fluorescently labeled dsRNA injected into the body cavity of the worm could itself be transported to the embryo (Chapter 3), it is unclear if dsRNA introduced outside the worm by soaking or feeding can reach progeny.

We began by soaking gravid adult worms in fluorescently-labeled *gfp*-dsRNA (see Fig. 3-1 for dsRNA schematic). After 3 hours of soaking, adult worms expressing *gfp* in oocytes and 1- and 2-cell embryos were not detectably silenced and dsRNA did not accumulate in oocytes, embryos, or coelomocytes (Fig. 6-9). The lack of detectable fluorescence in coelomocytes suggests that fluorescent dsRNA may not be reaching the body cavity after 3 hours of soaking and may require a longer soak. After soaking worms for 5 hours or 18 hours, dsRNA could be detected in coelomocytes (5 hours, data not shown; 18 hours, Fig. 6-10, *bottom*). dsRNA could be detected in oocytes and in some early embryos one of the two times worms were soaked for 18 hours (Fig. 6-11). However, oocyte morphology appeared slightly altered and only a few embryos contained dsRNA despite worms being soaked for 18 hours. In embryos where dsRNA could be detected, *oma-1::gfp* appeared to be

partially silenced (Fig. 6-11, *bottom*, compared to Fig. 6-9, *bottom*). Worms expressing *gfp* in early gut cells in the embryo (*Pend-1::gfp*) were also soaked in fluorescently labeled dsRNA for 18 hours but dsRNA was only weakly detected in some oocytes and never in embryos (data not shown). Together, these results suggest that dsRNA introduced outside the worm may reach progeny, however, the soaking RNAi assay does not consistently result in the detection of dsRNA in progeny. Future work is required to develop a reproducible soaking assay and to determine if the fluorescence detected in embryos is indeed dsRNA capable of silencing a gene.

Optimizing this soaking assay will allow dsRNA to be introduced to the extracellular space in a more high-throughput assay than injecting individual worms. This will be beneficial when attempting to collect large samples of embryos for RNA analysis (as described above).

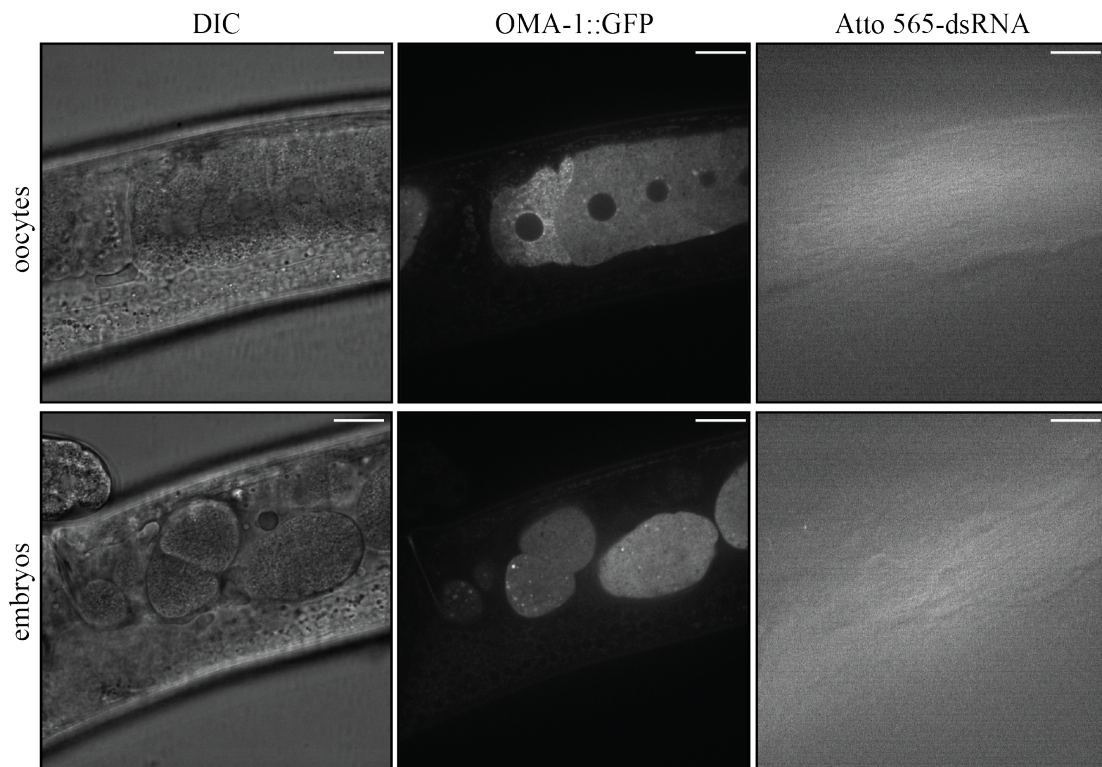


Fig. 6-9. Soaking worms in dsRNA for 3 hours does not result in the accumulation of dsRNA in oocytes, embryos, or coelomocytes. Adult worms expressing *Poma-1::oma-1::gfp* were soaked in Atto 565-labeled dsRNA for 3 hours and then imaged. Scale bars, 20 μ m; n = 4 worms.

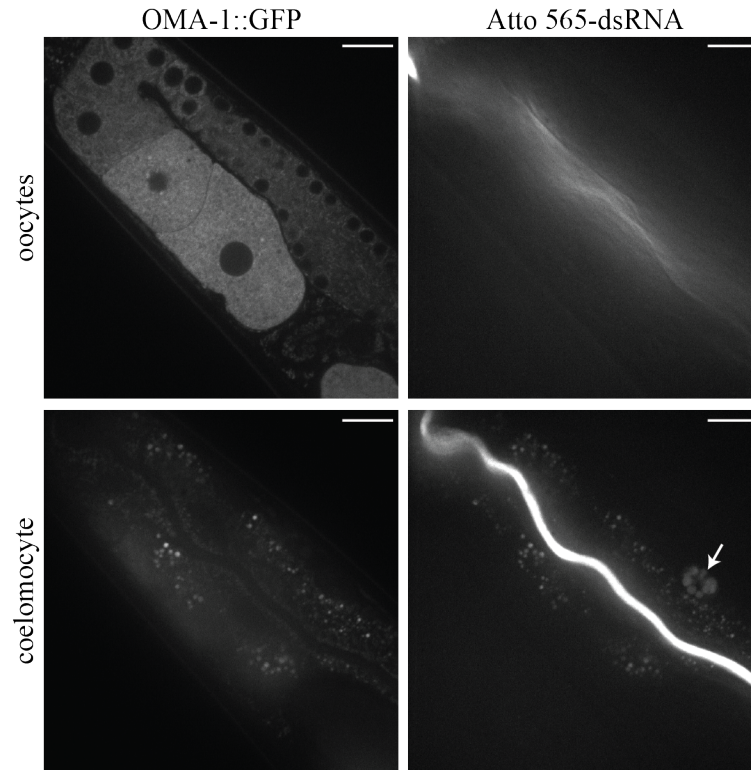


Fig. 6-10. First attempt at soaking worms in dsRNA for 18 hours resulted in the accumulation of dsRNA in coelomocytes and not in oocytes. Adult worms expressing *Poma-1::oma-1::gfp* were soaked in Atto 565-labeled dsRNA for 18 hours and then imaged. All 4 worms accumulated dsRNA in coelomocytes (representative image, *bottom*). White arrow indicates coelomocyte. 3/4 worms did not accumulate dsRNA in oocytes (representative image, *top*) while 1/4 worms displayed very mild accumulation of dsRNA in oocytes (not depicted). Scale bars, 20 μ m and n = 4 worms.

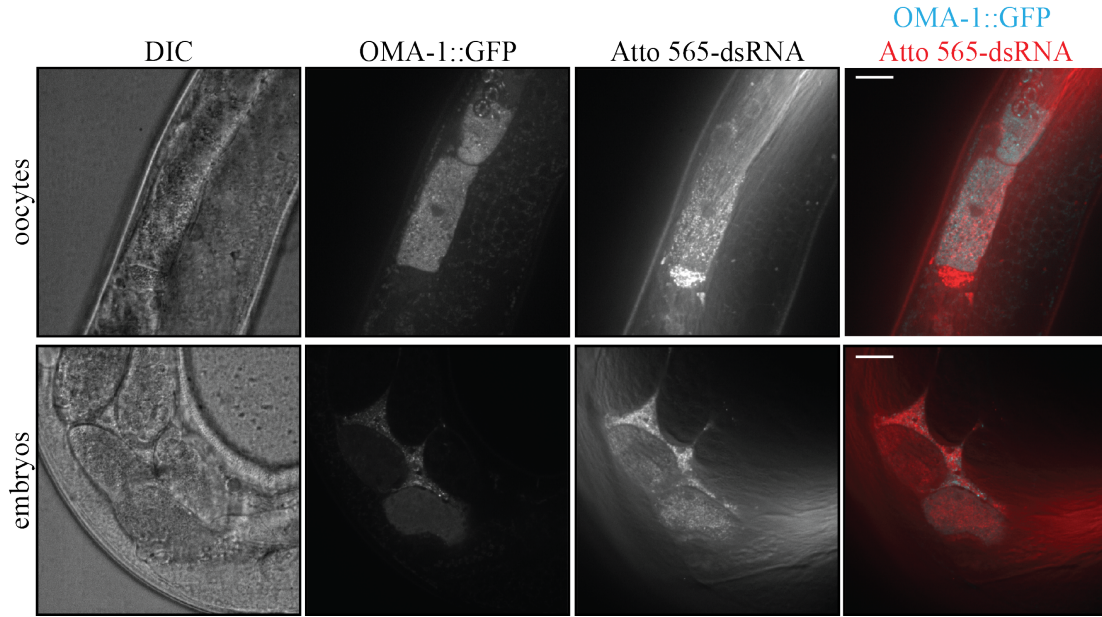


Fig. 6-11. Second attempt at soaking worms in dsRNA for 18 hours resulted in the accumulation of dsRNA in oocytes and embryos. Adult worms expressing *Poma-1::oma-1::gfp* were soaked in Atto 565-labeled dsRNA for 18 hours and then imaged. All 4 worms accumulated dsRNA in oocytes (representative image, *top*). 4 embryos from 2 worms accumulated dsRNA (representative image, *bottom*). Scale bars, 20 μ m.

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